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13. ABSTRACT ***(Maximum 200 Words)***

This grant addresses the molecular mechanisms of resistance to the anti-estrogen, Tamoxifen, in breast cancers. Both estrogens and antiestrogens influence the cell cycle during the G1 phase. In breast tumors, loss of the cdk inhibitor, p27, is associated with a poor prognosis and with steroid resistance. In the original proposal, we postulated that altered p27 degradation in breast cancer may contribute to resistance to antiestrogen therapy. We recently reported that the cdk inhibitors, p21 and p27 are essential for the G1 arrest of breast cancer cells by Tamoxifen. We have further discovered key molecular mechanisms that regulate how p27 is exported from the nucleus. We also reported that antiestrogen resistant cells show an increase in MAPK activation and inhibition of MAPK restores antiestrogen sensitivity. We published that oncogenic activation of MAPK changes p27 phosphorylation causing a loss in p27's cdk inhibitor function. Work of this last grant year has investigated further how p27 is regulated by the HER2 and MAPK pathways and how the export of p27 from the nucleus is linked to its ubiquitin-dependent degradation.

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Award Number: DAMD17-98-1-8158
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TITLE: Resistance to Tamoxifen: A Consequence of Altered $p27^{Kip1}$ Regulation During Breast Cancer

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REPORT DATE: June 2002

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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RESEARCH PROGRESS 2002
US Army Medical Research Command Award #DAMD17-98-8159

Grant Title: Resistance to Tamoxifen: a consequence of altered $p27^{Kip1}$ regulation during breast cancer progression

PI: J.M. Slingerland

INTRODUCTION

While approximately 70% of breast cancers express the estrogen receptor (ER) at diagnosis, only two thirds of these will respond to antiestrogens such as Tamoxifen (TAM). Unfortunately, ER positive tumors that are initially responsive, invariably acquire resistance to hormonal therapies (reviewed in (1)). TAM-resistant tumors usually show continued expression of the ER (2, 3). Estradiol regulates cell proliferation and development in the mammary gland. The elucidation of mechanisms whereby estradiol:ER influences cell cycle regulators and how these are blocked by Tamoxifen is highly relevant to the development of new treatments for steroid resistant breast cancer. Both estrogens and antiestrogens influence the cell cycle during the early G1 phase (4). The cell cycle is governed by a family of cyclin dependent kinases (cdks), whose activity is regulated by positive effectors, the cyclins, by phosphorylation and by negative regulators, the cdk inhibitors (reviewed in (5-7)). p27 or kinase inhibitor protein 1 (KIP1) is strongly expressed in normal mammary epithelial cells (8). That p27 protein levels are frequently reduced in primary breast cancers and this correlates with poor prognosis, suggests that p27 is an important negative regulator of the normal breast cell cycle (8-10) . The cellular abundance of p27 is importantly regulated by ubiquitin-mediated proteolysis (11). p27 protein decreases when quiescent MCF-7 breast cancer cells are stimulated to reenter the cell cycle with estradiol treatment and p27 increases when antiestrogens induce G1 arrest ((12, 13) and PNAS manuscript appended).

Hypothesis: When this grant was first submitted, we proposed the following **hypothesis** to be pursued by **3 specific aims**. We originally postulated that estrogen stimulates breast cancer cells to enter the cell cycle by activating p27 phosphorylation, thereby signaling its degradation. We also postulated that antiestrogens act to block p27 phosphorylation leading thereby to p27 accumulation and G1 arrest. Altered p27 degradation in breast cancer cells may underlie resistance to cytostasis by Tamoxifen. This hypothesis was to be pursued in the following Specific Aims.

AIM 1. We will compare effects of antiestrogen and estrogen on steroid sensitive and insensitive breast cancer lines which express the ER. We will test **a)** whether phosphorylation of p27 precedes the reduction in p27 levels following estradiol stimulation of steroid sensitive breast cancer lines; **b)** whether this is blocked by antiestrogens; and **c)** how estradiol sensitive and resistant lines differ in p27 protein expression, stability, p27 phosphorylation, localization and binding of novel p27-associated proteins.

AIM 2. To test whether increased ubiquitin proteasome activity lowers p27 levels in estradiol stimulated MCF-7 cells, we will use chemical proteosome inhibitors following estradiol stimulation of MCF-7; we will also use a temperature sensitive (ts) mutant of the ubiquitin activating enzyme E1 to determine how loss of this activity affects p27 levels, p27 phosphorylation and entrance into the cell cycle.

AIM 3. We will determine whether p27 is critical to growth inhibition by antiestrogens by introducing inducible antisense p27 into MCF-7 or by using antisense p27 oligonucleotides.

The original statement of work, SOW (proposed June 1997) was revised and this new SOW approved May 1998. The latter is added to the end of this progress report (see below). I will refer to both the work of the Specific Aims proposed above and the SOW TASKs throughout the Progress Report as appropriate.

REPORT BODY: SUMMARY OF PROGRESS ON GRANT

The work of Specific Aim 1a and all of Specific Aim 3 has been completed in the past 2 years and was published in 2000 in PNAS *USA* (***Appended Manuscript 1***). We have examined the effects of estradiol and antiestrogens on cell cycle regulators, including p27, in the MCF-7 line. We have shown that both p21 and p27 are essential mediators of the therapeutic effects of Tamoxifen and other antiestrogens. We have now published subsequent work supported by this grant that shows that deregulation of p27 by constitutive activation of the MAPK pathway can lead to Tamoxifen resistance (Donovan et al, Journal of Biologic Chemistry, 2001, ***Appended Manuscript 2***). Progress on Aim 1b and 1c that deals with how p27 phosphorylation and nuclear export are coordinated with its proteasomal degradation is presented below and will appear in Molecular Biology of the Cell in Jan 2003 (***Appended Manuscript 3***).

TASK 2, work completed for AIMs 1 and 3

p27 is required for the therapeutic effects of antiestrogens like Tamoxifen

The work of TASK 2 was in part accomplished and published in PNAS 2000. We found that estrogens and antiestrogens influence the G1 phase of the cell cycle. In MCF-7 breast cancer cells, estrogen stimulated cell cycle progression through loss of kinase inhibitor proteins (KIPs), p27 and p21, and G1 cyclin-cdk activation. Treatment with antiestrogen drugs, Tamoxifen or ICI 182780, caused cell cycle arrest, with up-regulation of both p21 and p27 levels, an increase in their binding to cyclin E-cdk2 and kinase inhibition. The requirement for p21 and p27 in the arrests induced by estradiol depletion or by antiestrogens was investigated using antisense. Antisense inhibition of p21 or p27 expression in estradiol-depleted or antiestrogen-arrested MCF-7 led to abrogation of cell cycle arrest, with loss of cyclin E-associated KIPs, activation of cyclin E-cdk2 and S phase entrance. These data demonstrate that depletion of either p21 or p27 can mimic estrogen-stimulated cell cycle activation and indicate that both of these KIPs are critical mediators of the therapeutic effects of antiestrogens in breast cancer. This was published in *PNAS* USA in 2000 (***Appended Manuscript 1***).

These data suggest that the reduced levels of p27 observed in human breast cancers may be linked to steroid independence. Our ongoing studies have addressed further the mechanisms whereby estradiol affects p27 action and how the regulation of p27 may be altered in steroid resistant breast cancer cells. See below.

TASK 2, work completed for AIMs 1c, and 2:

A Altered regulation of p27 and antiestrogen-resistance linked to increased MAPK activation in the steroid resistant MCF-7 cells (JBC 276(44): 40888-40895, ***Appended Manuscript 2).***

Work of the ***Appended Manuscript 2,*** together with that described above, **completes TASK2**. We have used the LY-2 cell line as a model to study antiestrogen resistance (15). Antiestrogens, such as Tamoxifen, are widely used to treat breast cancer. The therapeutic effects of antiestrogens require action of the cell cycle inhibitor $p27^{Kip1}$, leading to G1 arrest in estrogen receptor positive (ER+) breast cancer cells. Antiestrogen resistance in the LY-2 line is not due to mutation of the ER (3). Asynchronous Tamoxifen sensitive MCF-7 and the resistant LY-2 proliferate with similar percent S phase. Antiestrogen treatment of MCF-7 leads to cell cycle

arrest in MCF-7 but had very little effect on the cell cycle profile of LY-2, confirming that the LY-2 are antiestrogen resistant.

In the last year, we reported that constitutive activation of the mitogen activated protein kinase (MAPK) pathway alters p27 phosphorylation, reduces p27 protein levels, reduces the cdk2 inhibitory activity of the remaining p27, and contributes to antiestrogen resistance. In two antiestrogen resistant cell lines that showed increased MAPK activation, inhibition of the MAPK kinase (MEK) by addition of U0126 changed p27 phosphorylation and restored p27 inhibitory function and sensitivity to antiestrogens. Using antisense p27 oligonucleotides, we demonstrated that this restoration of antiestrogen-mediated cell cycle arrest required p27 function. These data suggest that oncogene-mediated MAPK activation, frequently observed in human breast cancers, contributes to antiestrogen resistance through p27 deregulation

B. How p27 regulation differs in steroid sensitive and resistant lines

How localization of p27 and its nuclear export and subsequent degradation are regulated have been assayed over the last year. We have prepared different phosphomutant vectors of p27 to address specifically how changes in p27 phosphorylation alter its ability to bind and inhibit cyclin E-cdk2 and inhibit cell cycle progression, and how p27 phosphorylation affects its import and export from the nucleus and degradation. These experiments represent the continuation of the work proposed in AIM1c and 2. AIM 1c proposed an investigation of the relationship between p27 localization and stability. The sites in p27 that are regulated by the MEK/MAPK pathway are under investigation.

Investigations in our lab and others have shown that in addition to the "classic" cyclin E-cdk2/SKP2 mediated degradation of p27 in late G1, there appears to be a second mechanism activating p27 proteolysis in early G1. In early G1, mitogens sitmulate a change in p27, possibly through phosphorylation, that lead to p27 export and proteolysis. This facilitates activation of cyclin E-cdk2 in late G1. Cyclin E-cdk2, once activated, can then bind and phosphorylated p27 at threonine 187 leading to its ubiquitylation the SKP2 ubiquitin ligase. We have new data now that suggests that the oncogenic activation of receptor typrsine kinase (RTKs), leads to hyperactive MAPK signaling and this leads to accelerated p27 proteolysis.

We have confirmed that MAPK activation following transfection of activated MAPK kinase (MEK^{EE}), leads to a shortening of the half-life of p27. In addition MAPK shifts the phosphorylation of p27 protein to a different pattern on two-dimensional isoelectric focusing. MEK^{EE} overexpression and MAPK activation cause a shift of p27 out of the nucleus and into the cytoplasm and the cytoplasmic p27 is hyperphosphorylated compared to that in cells without constitutive MAPK activation. This work, that shows how mitogenic signaling via MAPK inactivates p27's cell cycle inhibitory function through an increase in its proteolysis, is currently in preparation fro publication.

TASK 1 (see revised SOW at end of this progress report)

p27 Export is regulated by p27 phosphorylation and by binding to CRM1 and RanGTP (Molecular Biology of the Cell in press, manuscript appended, ***Appended Manuscript 3)***

The work of the revised TASK1 (Aim1c) has now been completed. In pursuit of Specific AIM 1c, our immunofluorescence studies revealed that the localization of p27 changes across the cell cycle. Our preliminary data suggested that export of p27 from nucleus to cytoplasm precedes its degradation. Recent work by others also suggests a connection between nuclear transport and p27 degradation (14). In some breast cancers progression to estrogen

independence may be linked to accelerated export-dependent p27 proteolysis. In the work of the past 4 years, we have shown that nuclear export of p27 is critically regulated by phosphorylation events that mediate p27's association with components of the nuclear export machinery and its subsequent degradation. The following paragraph summarizes our studies on nuclear export of p27, that have been presented a two international Cell Cycle meetings over the last 2 years in May, 2000 and Jan, 2001. This work has been published on line in Molecular Biology of the Cell in Oct 2002 and will be in print Jan 2003. For greater detail, see the manuscript appended.

p27 regulates G_1 entry by inhibiting cyclin E/cdk 2 activity, and cellular p27 protein levels are regulated primarily by proteasomal degradation. Ubiquitin-mediated degradation of p27 in late G1 requires phosphorylation on threonine 187 (T187-P) and p27 binding to cyclin E/cdk 2 or cyclin A/cdk 2. p27 degradation involves an SCF complex, which contains SKP2, SKP1, Cul1 and Ubc3. Earlier work suggested that p27 degradation is inhibited by leptomycin B (LMB), a cytotoxin that disrupts the interactions between the nuclear export protein CRM1 and the nuclear export sequence (NES) of proteins to be exported. Therefore, we investigated the mechanisms regulating p27 nuclear export across the cell cycle.

We have shown that p27 localization is cell cycle regulated and that active CRM1/RanGTP-mediated nuclear export of p27 may be linked to cytoplasmic p27 proteolysis in early G1. p27 is nuclear in G0 and early G1 and appears transiently in the cytoplasm at the G1/S transition. Co-precipitation of p27 with the exportin CRM1 was minimal in G0 and p27-bound CRM1 increased markedly during G1-to-S phase progression. Proteasome inhibition in mid G1 did not impair nuclear import of p27, but led to accumulation of p27 in the cytoplasm, suggesting that export precedes degradation for at least part of the cellular p27 pool. p27-CRM1 binding and nuclear export were inhibited by mutation of serine 10 to alanine (S10A) mutation but not by T187A mutation. A putative nuclear export signal (NES) in p27 was identified whose mutation reduced p27-CRM1 interaction, nuclear export and p27 degradation. LMB did not inhibit p27-CRM1 binding *in vitro* or *in vivo*, nor did it prevent p27 export *in vitro* or in heterokaryon assays. Pre-binding of CRM1 to the classical NES of HIV-1 Rev did not inhibit p27-CRM1 interaction, suggesting that p27 binds CRM1 at a non-LMB sensitive motif. LMB increased total cellular p27 and may do so indirectly though effects on other p27 regulatory proteins. These data and other recent work suggest a model in which p27 undergoes active, CRM1-dependent nuclear export and cytoplasmic degradation in early G1. This would permit the incremental activation of cyclin E-Cdk2 leading to cyclin E-Cdk2 mediated T187 phosphorylation and p27 proteolysis in late G1 and S phase.

During the course of this year, I have acknowledged the support from the US Army Career Development Award in all publications coming out of my lab even if the projects were not directly supported by the original grant proposal (also funded as an Ideas award 1998-2001). This acknowledgement reflects the salary support provided to me by the DOD. I have included these papers in the Reportable Outcomes section of this progress report and appended these papers as ***Appended Manuscripts 4-6.***

MENTORING

This DOD grant has supported a PhD trainee, Mr. Jeff Donovan, and a post-doctoral research fellow, Dr. M. Connor. Dr Donovan was initially supported by the IDEAS award from the DOD *DAMD17-98-8158* (same proposed grant project as was supported by my Career Development Award by the DOD # *DAMD17-98-8159.* He subsequently applied for and received a DOD pre-doctoral breast cancer research fellowship. Mr. Donovan is co-first author on the research article

that we published in PNAS (appended) and has now published second paper in JBC on the work of this grant (appended). He is first author on the JBC paper. He is an MD/PhD and plans to pursue an academic career as a clinician investigator. He completed his PhD defense Aug 16th, 2002. As of Sept 2002, he resumed his medical degree studies at the University of Ottawa. During his time in my lab, he has become interested in having an academic career in Medical Oncology. I am convinced Jeff has what it takes to marry a research and clinical career. Jeff has a strong interest in a career in which he pursues the application of molecular biologic techniques to solve real therapeutic problems in the practice of breast Oncology.

Dr. Connor joined the lab in August of 2000. He carried on the work that a former post-doctoral fellow Dr. S. Cariou had begun. Dr. Connor has been very productive during his time in my laboratory. In December, Dr. Connor was joined by another post-doctoral fellow, Dr. R. Kotchetkov, in the study of how the nuclear export of p27 is regulated. Together these two postdocs have uncovered important mechanisms whereby p27 export from the nucleus regulates p27 degradation. This has been an international collaboration with colleagues at the Max Planck Institute in Germany and the work will appear in the Jan 1, 2003 edition of Molecular Biology of the Cell (appended). Dr. Connor hopes to obtain a position as an independent investigator in the cancer research field when he completes his post-doctoral training. .

Dr Angel Arnaout is a Surgical Resident who recently joined my lab. She is interrupting her residency training in General Surgery to carry out a Masters degree. She will complete her MSc at the University of Toronto in the spring of 2003 in the molecular biology of breast cancer. Dr Arnaout intends to pursue a career in Surgical Oncology with a focus on breast cancer surgery. She has been a pleasure to mentor as she prepares for a career in translational research in the field of Surgical Oncology, with a focus on breast cancer. As she completes her graduate work at the U of Toronto she is being co-mentored by 2 other U of T faculty, and I continue to supervise her training from Miami.

Jeff Rothenstein worked in my lab over the summer of 2001 on the DOD project after completing his first year as a medical student. This has provided him valuable experience in translational research applied to breast cancer. While Kathy Han did not work on the DOD breast cancer project, she is also a summer student who worked with me over the last two years and has now entered Medical School at McGill University, as of Sept 2002. She will likely go on to have a career as a Clinical Scientist since she is a very able researcher. Albert Chang worked for a year in my lab as a Technician between Aug 2001 and Aug2002. He had completed an MSc in molecular biology. He entered Medical School in Sept 2002 and I believe that he also intends to pursue a career as a Clinical Scientist on completion of his medical degree. Helen Chan worked in my lab between Feb 2002 and Aug2002 after completing her MSc in Feb 2002 at another University. She is a talented molecular biologist and enjoyed working in my lab as it gave her the flavor of what translational research was like. She entered Medical School at Queens University in Sept 2002 and also intends to pursue a career in translational research.

CAREER IMPACT

This CAREER DEVELOPMENT AWARD for the DOD Breast Cancer Research Program *DAMD17-98-8159* provided the support required for an increase in the proportion of my time devoted to laboratory research. My breast cancer patients have been most heartened to hear that I

am supported by such a generous grant from the DOD. They often ask me how my research is going and are very supportive.

In 2001, I was awarded a Canada Research Chair. This is an honor and also provides some career support. I think the DOD awards I have received have been helpful not only in the material support it has provided for lab operations, but also in helping to establish my credibility as a Clinician Scientist. The DOD grants IDEAS #DAMD17-98-8158 and Career Development #DAMD17-98-8159 have also provided support for the translational research work that led U of Miami to recruit me to their direct their breast cancer program. I moved to U of Miami in September 2002 to take up the position of Director of the Braman Breast Cancer Research Institute. This Multidisciplinary research institute will attempt to develop, expand and bring together research endeavors on breast cancer at the U of Miami. I am currently recruiting a Clinical Director, 4 other Clinical Faculty devoted to the breast program (Medical Oncologists, a breast Radiologist, and breast Surgeon) in addition to 4 other scientists investigating molecular mechanisms underlying breast cancer. I will be recruiting a Molecular Pathologist and a Molecular Epidemiologist and together we will establish an ongoing prospective breast tumor data-base and tumor bank here at U of Miami. We aim to not only provide exemplary clinical care of breast patients at our center, but also to carry out Clinical research in every aspect of breast cancer care, including breast imaging, less invasive breast cancer surgery, new methods of breast irradiation and to offer our patients the latest molecular based therapies as we expand phase one and two clinical trials of molecular targeted cancer therapies. The 4 scientists to be recruited to the program will be investigating molecular mechanisms of breast cancer that can then be validated in human breast tumor samples from the data bank. Once new molecular targets have been validated, we will move these targets toward drug development and Phase I trials in the nearest possible time frame. The Braman breast Cancer Institute will also provide a forum for dialogue between the many other scientists at the UM who are doing work of relevance to the molecular biology of carcinogenesis and cancer progression.

Revision of SOW for grant award numbers DAMD17-98-1-8158 and DAMD17-98-1-8159 (Approved in 1999)

PI: J. M. Slingerland

TASK 1 (revised)

1) Assay effects of LlnL on p27 levels and its intracellular localization (year 1, done)

2) Carry out nuclear cytoplasmic fractionation and test whether p27 phosphorylation differs in the cytoplasmic and nuclear fractions using 2 dimensional immunoelectrophoresis techniques that have been established in the Slingerland lab (done in year 2)

3) Test whether in vivo associations between p27 and components of the nuclear export machinery (export carrier proteins, CRM1, importin beta, transport proteins Ran and RanGAP) are detectable and whether they vary across the cell cycle. (8 months required in year 2, completed)

4) Test the effects of inhibition of the proteasome on the levels of p27 binding to nuclear export factor CRM1 (2-3 months in year 2, completed).

5) Show whether the ability of p27 immunoprecipitated from MCF-7 to bind recombinant CRM1 varies as a function of cell cycle progression and is associated with differences in p27 phosphorylation (completed in year 2).

6) Investigate how MCF-7 variants that have become steroid independent differ in their regulation of p27 localization and proteolysis (year 2 and 3, work underway).

TASK 2 remains unchanged and will be completed by the end of the grant period

List of Key Accomplishments over the whole grant period 1998-2002

1. We have shown that both p21 and p27 are essential mediators of the therapeutic effects of Tamoxifen and other antiestrogens

2. In two antiestrogen resistant cell lines with increased MAPK activation , we showed that inhibition of the MAPK kinase (MEK) by addition of U0126 changed p27 phosphorylation and restored p27 inhibitory function and sensitivity to antiestrogens

3. We demonstrated that constitutive activation of the mitogen activated protein kinase (MAPK) pathway alters p27 phosphorylation, reduces p27 protein levels, reduces the cdk2 inhibitory activity of the remaining p27, and contributes to antiestrogen resistance

4. Our data suggest that oncogene-mediated MAPK activation, frequently observed in human breast cancers, contributes to antiestrogen resistance through p27 deregulation

5. Demonstrated that for a portion of cellular p27, export of p27 from nucleus to cytoplasm precedes its degradation. We have shown that p27 localization is cell cycle regulated and that active CRM1/RanGTP-mediated nuclear export of p27 is linked to cytoplasmic p27 proteolysis in early G1.

6. A putative nuclear export signal (NES) in p27 was identified whose mutation reduced p27-CRM1 interaction, nuclear export and p27 degradation

7. We have confirmed that MAPK activation following transfection of activated MAPK kinase (MEK^{EE}), leads to a shortening of the half-life of p27.

8. We showed that MAPK shifts the phosphorylation of p27 protein to a different pattern on two-dimensional isoelectric focusing. MEK^{EE} overexpression and MAPK activation cause a shift of p27 out of the nucleus and into the cytoplasm and the cytoplasmic p27 is hyperphosphorylated compared to that in cells without constitutive MAPK activation. These changes are key to the MAPK mediated p27 proteolysis.

List of Reportable Outcomes 2002

Manuscripts

Publications:

1. Cariou, S., Donovan, J.C.H., Flanagan, W.M., Milic, A., Bhattacharya, N., **Slingerland, J.M.** (2000) Downregulation of $p21^{WAF1/Cip1}$ or p^{27K1p1} abrogates antiestrogen mediated G1 arrest in human breast cancer cells. **Proceedings of the National Academy of Science (USA),** 97(16):9042-9046. (Corresponding author, 100% effort in my lab) SEE APPENDED MANUSCRIPT 1

2. Donovan JCH, Milic A, **Slingerland J.** (2001) Activation of p42/44 MAPK pathway leads to $p27^{Kip1}$ deregulation and antiestrogen resistance in human breast cancer cells. **Journal of Biological Chemistry**, 276(44): 40888-40895 (Corresponding author, 100%effort) SEE APPENDED MANUSCRIPT 2

3. Connor, M., Kotchetkov, R., Carious, S., Resch, A., Lupetti, R., Beniston, R., Melchior, F., Hengst, L., **Slingerland, J.** (2002) Nuclear export of $p27^{Kip1}$ is regulated by phosphorylation and by RanGTP/CRM1 binding. In Press **Molecular Biology of the Cell** (published on line Oct, 2002, Corresponding author, 90% in my lab). SEE APPENDED MANUSCRIPT 3

4. Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M., Han, K., Lee, J.-W., Ciarallo, S., Catzavelos, C., Franssen, E., Beniston, R., **Slingerland, J.** (2002) Phosphorylation of $p27^{Kip1}$ by PKB/Akt leads to $p27^{Kip1}$ mislocalization and TGF-β resistance. **Nature Medicine** 8 (10): 1153-1160 (Corresponding author, 100% effort my lab) SEE APPENDED MANUSCRIPT 4

5. Ciarallo, S., Subramaniam V., Kotchetkov, R., Lee, J.H., Sandhu, C., Milic, A., **Slingerland, J.M.** (2002) Altered $p27^{kip1}$ phosphorylation, localization and function in human mammary epithelial cells resistant to TGF-β mediated G1 arrest. **Molecular and Cellular Biology**, 22(9): 2993-3002, (Corresponding author, 100% effort from my lab). SEE APPENDED MANUSCRIPT 5

6. Donovan, J., Milic, A. Rosenberg, J. and **Slingerland, J.** (2002) The requirement for p27 in G1 arrest by TGF-β becomes essential during progressive checkpoint loss in human cancers. In Press **Journal of Biological Chemistry**, (published on line Sept, 2002. (Corresponding author, 100% effort) SEE APPENDED MANUSCRIPT 6

Abstracts and Presentations

Plenary Talks at International Meetings:

1. **Slingerland JM**: The MAPK pathway: new molecular targets for the therapy of hormone resistant breast cancer. 4rth International Conference on Sex and Gene Expression, San José, April 5-7, 2002. ABSTRACT APPENDED

2. Jiyong Liang, **Joyce M. Slingerland**: Akt/PKB-dependent phosphorylation of p27 activates the cyclin D1/Cdk4 assembly function of p27 and G1 cell cycle progression. The Cell Cycle , Cold Spring Harbor, May 17, 2002. ABSTRACT APPENDED

Invited Lectures:

1. **Slingerland JM**: Mechanisms of resistance to antiestrogen therapy in breast cancer: new molecular insights. The ECOG Annual Meeting, Miami, FL, Nov 18, 2002

2. **Slingerland JM**: PKB phosphorylates p27 and leads to its cytoplasmic mislocalization – relevance to human breast cancer. Research Seminar, Palo Alto Medical Foundation, Stanford University, California, April 4, 2002.

3. **Slingerland JM**: PKB phosphorylates p27 and leads to its cytoplasmic mislocalization – relevance to human breast cancer. Research Seminar, University of California at San Francisco, California, April 2, 2002.

4. **Slingerland JM**: Cytoplasmic mislocalization of the cdk inhibitor p27: links to oncogenic PKB activity and prognostic import in human breast cancer. Research Seminar, Baylor College of Medicine, Houston, Texas, February 4, 2002.

5. **Slingerland JM**: Degradation or Mislocalization of the cdk Inhibitor p27: Prognostic Import in Human Breast Cancer. Oncology Grand Rounds, Rochester Cancer Center, Rochester, New York, January 27, 2002.

6. **Slingerland JM**: Deregulation of the cell cycle inhibitor p27 in human breast cancer. Oncology Rounds The Sylvester Comprehensive Cancer Centre, University of Miami School of Medicine, Miami, Florida, November 27th, 2001.

7. **Slingerland JM**: p27^{kip1}, a Novel Prognostic Factor and Predictor of Tamoxifen Responsiveness in Breast Cancer. Cancer Education Seminars, Stanford University Medical Centre, Palo Alto, California, August 14th, 2001.

Funding applied for based on work supported by this award:

Pre-doctoral Fellowship Award Submitted by J. Donovan to US Army MRMC Breast Cancer Program, June 2, 1999 and awarded Jan, 2000-2003.

Degrees obtained under support of this award:

Jeff Donovan (MD-PhD student supported by this grant) completed his Ph.D. thesis defense on August 16, 2002. He has returned to the University of Ottawa to complete his MD degree.

Conclusions

CLINICAL IMPLICATIONS OF WORK COMPLETED

The work completed on TASKS 1 & 2 have several important clinical implications. The humanized antibody to HER2, rhuMAbHER2 (Trastuzumab) has therapeutic efficacy in the treatment of HER2 overexpressing breast cancers (16). In patients with ER+ breast cancers that are responding to Trastuzumab, the MAPK downregulation afforded by inhibition of HER2 signaling may restore Tamoxifen sensitivity. The potential for additive therapeutic effects of Tamoxifen in Trastuzumab (Herceptin) responders warrants testing in clinical trials. Moreover, tyrosine kinase inhibitors such as Emoda, which block HER-2 phosphorylation and its intracellular signaling, may restore or extend the period of responsiveness to Tamoxifen.

Although up to 60% of newly diagnosed breast cancers express the ER, only about 2/3 of these respond to antiestrogens like Tamoxifen. A second clinical implication of this study is that low p27 levels in ER+ breast cancers may predict for primary non-responsiveness to Tamoxifen. We are currently investigating this in a large retrospective study of 800 patients treated with Tamoxifen in the 1980s. Moreover, novel MAPK inhibitor drugs (17) could have the potential to restore steroid responsiveness in ER+ hormone resistant cancers.

Insights into the regulation of p27 localization and degradation are germane to the loss of p27 protein seen in primary breast cancer. In primary breast cancers, there is a strong association between reduced p27 protein levels and progression to steroid independence (8). We have shown, in work supported by this grant, that p27 is essential for the maintenance of growth arrest of breast cancer cells following Tamoxifen treatment or interruption of estradiol signaling. A better understanding of how this key effector, p27, is regulated and how these processes are altered in breast cancer progression has shed new insights on the problem of progression to Tamoxifen resistance in breast cancer and provided a rationale for the development of new clinical trials combining antiestrogen therapies with specific receptor tyrosine kinase inhibitors, such as Iressa or Herceptin with antiestrogens such as Tamoxifen, Arimades or Letrozole.

References

1. Brown, M. Estrogen receptor molecular biology. Hematology/Oncology Clinics of North America, *8*: 101-112, 1994.
2. Robertson, J.F.R. Oestrogen receptor: a stable phenotype in breast cancer. British Journal Of Cancer, *73*: 5-12, 1996.
3. Mullick, A. and Chambon, P. Characterization of the strogen receptor in two antiestrogn-resistant cell lines, LY2 and T47D. Cancer Research, *50*: 333-338, 1990.
4. Musgrove, E.A. and Sutherland, R.L. Cell cycle control by steroid hormones. Cancer Biology, *5*: 381-389, 1994.
5. Morgan, D.O. Principles of Cdk regulation. Nature, *374*: 131-134, 1995.
6. Sherr, C.J. G1 phase progression: cycling on cue. Cell, *79*: 551-555, 1994.
7. Sherr, C.J. and Roberts, J.M. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev., *9*: 1149-1163, 1995.
8. Catzavelos, C., Bhattacharya, N., Ung, Y.C., Wilson, J.A., Roncari, L., Sandhu, C., Shaw, P., Yeger, H., Morava-Protzner, I., Kapusta, L., Franssen, E., Pritchard, K.I., and Slingerland, J.M. Decreased levels of the cell cycle inhibitor p27Kip1 protein: prognostic implications in primary breast cancer. Nature Med., *3*: 227-230, 1997.
9. Tan, P., Cady, B., Wanner, M., Worland, P., Cukor, B., Magi-Galluzzi, C., Lavin, P., Draetta, G., Pagano, M., and Loda, M. The cell cycle inhibitor p27 is an independent prognostic marker in small (T1a,b) invasive breast carcinomas. Cancer Res., *57*: 1259-1263, 1997.
10. Porter, P.L., Malone, K.E., Heagerty, P.J., Alexander, G.M., Gatti, L.A., Firpo, E.J., Daling, J.R., and Roberts, J.M. Expression of cell cycle regulators p27kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. Nature Med., *3*: 222-225, 1997.
11. Pagano, M., Tam, S.W., Theodoras, A.M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P.R., Draetta, G.F., and Rolfe, M. Role of ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science, *269*: 682-685, 1995.
12. Foster, J. and Wimalasen, J. Estrogen regulates activity of cyclin-dependent kinases and retinoblastoma protein phosphorylation in breast cancer cells. Mol.End., *10*: 488-96, 1996.
13. Watts, C.K.W., Brady, A., Sarcevic, B., deFazio, A., and Sutherland, R.L. Antiestrogens inhibition of cell cycle progression in breast cancer cells is associated with inhibition of cyclin-dependent kinase activity and decreased retinoblastoma protein phosphorylation. Mol.End., *9*: 1804-13, 1996.
14. Tomoda, K., Kubota, Y., and Kato, J. Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1. Nature, *398*: 160-165, 1999.
15. Bronzert, D.A., Greene, G., and Lippman, M.E. Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY 117018. Endocrinology, *117*: 1409-1417, 1985.
16. Kurebayashi, J.J. Biological and Clinical Significance of HER2 Overexpression in Breast Cancer. Breast Cancer, *8*: 45-51, 2001.
17. Sebolt-Leopold, J.S., Dudley, D.T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R.C., Tecle, H., Barrett, S.D., Bridges, A., Przybranowski, S., Leopold, W.R., and Saltiel, A.R. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat Med, *5*: 810-816, 1999.
18. Slingerland, J. and Pagano, M. Regulation of the cdk inhibitor p27 and its deregulation in cancer. J Cell Physiol, *183*: 10-17, 2000.
19. Loda, M., Cukor, B., Tam, S.W., Lavin, P., Fiorentino, M., Draetta, G.F., Jessup, J.M., and Pagano, M. Increased proteasome-dependent degradation of the cyclin- dependent kinase inhibitor p27 in aggressive colorectal carcinomas. Nature Med., *3*: 231-234, 1997.

Down-regulation of $p21^{WAF1/CIP1}$ or $p27^{Kip1}$ abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells

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Estrogens and antiestrogens influence the G_1 phase of the cell cycle. In MCF-7 breast cancer cells, estrogen stimulated cell cycle progression through loss of the kinase inhibitor proteins (KIPs) p27 and p21 and through G_1 cyclin-dependent kinase (cdk) activation. Treatment with antiestrogen drugs, Tamoxifen or ICI 182780, caused cell cycle arrest, with up-regulation of both p21 and p27 levels, an increase in their binding to cyclin E–cdk2, and kinase inhibition. The requirement for these KIPs in the arrests induced by estradiol depletion or by antiestrogens was investigated with antisense. Antisense inhibition of p21 or p27 expression in estradiol-depleted or antiestrogen-arrested MCF-7 led to abrogation of cell cycle arrest, with loss of cyclin E-associated KIPs, activation of cyclin E–cdk2, and S phase entrance. These data demonstrate that depletion of either p21 or p27 can mimic estrogen-stimulated cell cycle activation and indicate that both of these KIPs are critical mediators of the therapeutic effects of antiestrogens in breast cancer.

Estradiol is mitogenic in up to 50% of *de novo* breast cancers, causing recruitment of quiescent cells into G_1 and shortening the G_1-to-S phase interval (1, 2). Although 70% of breast cancers express the estrogen receptor (ER), only two-thirds of these will respond to antiestrogens, of which, Tamoxifen is the most widely used (3, 4). Antiestrogens, such as Tamoxifen, its active metabolite, 4-hydroxytamoxifen (4-OH TAM), and the more potent steroidal antiestrogen ICI 182780 (Faslodex) lead to a G_0/G_1 arrest in susceptible ER-positive breast cancer cells (5–8). Unfortunately, hormonally responsive breast cancers invariably develop resistance to antiestrogens despite the continued expression of wild-type ER in most cases (9–12). Estrogens induce conformational changes in the ER, which promote its nuclear localization, dimerization, and function as a ligand-activated transcription factor (13–15). In addition, ligand binding to the ER can rapidly and transiently activate signal transduction pathways, notably the mitogen-activated protein kinase in breast cancer and in other cell types (16, 17). Because antiestrogen resistance usually develops in the presence of an intact ER, the mechanisms by which ER modulates the cell cycle may be altered during breast cancer progression. The evolution of prostate cancer to hormone independence also occurs without loss of the androgen receptor (18, 19) and may reflect a common mechanism of cell cycle misregulation.

Progression through the cell cycle is governed by a family of cyclin-dependent kinases (cdks), whose activity is regulated by phosphorylation (20), activated by cyclin binding (21, 22), and inhibited by the cdk inhibitors of the inhibitor of cdk4 (INK4) family ($p16^{INK4A}$, $p15^{INK4B}$, p18, and p19) and kinase inhibitor protein (KIP) family ($p21^{WAF-1/CIP-1}$, $p27^{Kip1}$, and $p57^{KIP2}$; refs. 22–24). Passage through G_1 into S phase is regulated by the activities of cyclin D-, cyclin E-, and cyclin A-associated kinases. Although p27 protein is strongly expressed in normal mammary epithelial tissue, decreased levels of p27 protein in primary breast cancers are correlated with poor prognosis (25, 26) and steroid independence (25). Reduced p21 levels have also been associated with a poor prognosis in some breast cancer studies (27–29). Expression of the ER, a good prognostic factor in breast cancer, is associated with higher levels of both p21 and p27 proteins (25, 27, 28, 30). Our observation that loss of p27 was strongly associated with hormone independence (25) stimulated the present investigation of the role of these KIPs in cell cycle effects of estrogen and antiestrogens in breast cancer cells.

Although recent reports correlate estrogenic stimulation with activation of cyclin E–cdk2, some suggest the importance of the cdk inhibitor p21 (31, 32) and others emphasize a role for p27 (33). An understanding of how estrogens and antiestrogens influence the cell cycle and the mechanisms of their alteration in cancer progression may facilitate the development of new hormonal treatments for breast cancer and other hormone-dependent cancers. The present study provides evidence that both p21 and p27 play essential roles in the cell cycle arrest of breast cancer cells by antiestrogens.

Materials and Methods

Cell Culture and Synchronization. MCF-7 cells (34) were grown in improved modified essential medium (IMEM-option Zn^{2+}) supplemented with insulin and 5% (vol/vol) FCS. Cells were transferred to phenol red-free medium for 48 h and then synchronized in quiescence by depletion of estradiol through transfer to IMEM-option Zn^{2+} supplemented with 5% (vol/vol) charcoal-stripped FCS for 48 h.

Analysis of Cell Cycle Regulators. Cells were released from quiescence by readdition of 10 nM 17-β-estradiol (estradiol) at $t = 0$ h. At intervals thereafter, cells were harvested for cell cycle analysis by flow cytometry after BrdUrd pulse labeling and propidium iodide counterstaining (35) and for Western analysis of cyclins E, D1, A, and B, as well as cdk2, cdk4, cdk6, p15, p21, and p27 proteins (from 20–50 μg of protein extract) as described (36). Equal protein loading was verified by probing these blots with antibody to β-actin (Sigma). Quantitation of proteins on Western and immunoprecipitation–Western blots was done by densitometry with IMAGEQUANT software. Cyclin E was immunoprecipitated from 100–150 μg of protein lysate with anti-cyclin E mAb 172 and associated proteins detected by immunoblotting or associated kinase assayed with histone H1 as substrate as described (36). At the times indicated,

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Abbreviations: cdk, cyclin-dependent kinase; KIP, kinase inhibitor protein; 4-OH TAM, 4-hydroxytamoxifen; INK4, inhibitor of cdk4; ER, estrogen receptor.

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Cdk4 immunoprecipitates (polyclonal antibody from Santa Cruz Biotechnology) were analyzed for associated proteins, and cyclin D1 was immunoprecipitated from 200 μg of protein with DCS-11 antibody (Neomarkers, Freemont, CA) for kinase assays with pRb substrate as described (37). Immunohistochemical analysis of p27 was undertaken at the indicated times after addition of estradiol as described (25).

Analysis of Cell Cycle Arrest by Inhibition of ER Signaling. Asynchronous MCF-7 cultures were either depleted of estradiol as described above or arrested by addition of 1 μM 4-OH-TAM (Sigma) or 10 nM ICI 182780 {7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]estra-1,3,5(10),-triene-3,17β-diol; Zeneca Pharmaceuticals, Wilmington, DE} to complete medium, and samples were collected at 0, 12, 18, 24, and 48 h thereafter for protein and flow cytometric analyses. p21, p27, cyclins E, and D1 proteins were assayed by Western analysis, and cyclin E-associated cdk2, p21, p27, and histone H1 kinase activities were assayed as in ref. 36.

Antisense Oligonucleotide Transfections. Phosphorothioate oligonucleotide sequences were as follows: GS5422 antisense p27 (ASp27), 5′-TGGCTCTCXTGCGCC-3′; GS5585 mismatch p27 (MSMp27), 5′-TGGCTCXCTTGCGCC-3′; GS6008 antisense p21 (ASp21), 5′-TCCGXGCCCAGCTCC-3′; GS6074 mismatch p21 (MSMp21), 5′-TCCGXCGCCAGCTCC-3′. X indicates the G clamp modification of these oligonucleotides (38). MCF-7 cells rendered quiescent by estradiol depletion or by treatment with 10 nM ICI 182780 or 1 μM 4-OH TAM were transfected with 120 nM oligonucleotides by using 2.5 μg/ml cytofectin G3815 (Gilead Scientific, Foster City, CA) for 6 h as described (38, 39), followed by replacement with complete medium. Flow cytometry and protein analysis were performed at 1, 21, and 28 h thereafter. Neither p21 nor p27 was increased in mismatch controls compared with lipid-only transfections.

Metabolic Labeling. Before and at 1 h and 21 h after completion of antisense oligonucleotide transfection, cells were metabolically labeled, followed by immunoprecipitation of p21 and p27. Cells were incubated for 30 min in 8 ml of α-MEM lacking methionine and then labeled metabolically for 1 h with 500 μCi [^{35}S]methionine in 2 ml of α-MEM lacking methionine. Cells were lysed on ice, clarified by centrifugation, and precleared with 1 μg of nonspecific rabbit polyclonal IgG and protein A Sepharose beads. [^{35}S]Methionine incorporation was quantitated as trichloroacetic acid-insoluble counts. Lysates volumes representing equal amounts (10^8 cpm) of total trichloroacetic acid-incorporated counts were immunoprecipitated for 1 h with either p21 or p27 antibody or with control nonspecific rabbit polyclonal IgG. Immune complexes were collected on protein A Sepharose beads, washed three times, and eluted into Laemmli buffer. Samples were resolved by SDS/PAGE; gels were dried; and labeled proteins were visualized by autoradiography.

Results and Discussion

Estradiol stimulates a shift of KIP proteins from cyclin E–cdk2 into cyclin D1–cdk4. Estradiol stimulation of steroid-deprived, quiescent MCF-7 breast cancer cells induced synchronous cell cycle reentry. S phase entrance was detected by 12 h, with the peak percentage of S phase cells at 24 h (Fig. 1*A*). The levels of cyclin E, cdk2, and cdk4 remained constant, and p15^{INK4B} protein levels fell as cells moved into G_1 (data not shown). MCF-7 cells do not express p16^{INK4A} because of deletion of this gene (40). Cyclin D1 was not detected in quiescent cells but rose within 3 h of estradiol addition and remained constant thereafter. p21 and p27 protein levels fell by 3- and 5-fold, respectively, by 24 h (Fig. 1*B*). Immunohistochemical analysis of p27 supported the immunoblotting data (Fig. 1*C*). Estradiol-depleted, quiescent MCF-7 cells showed strong nuclear

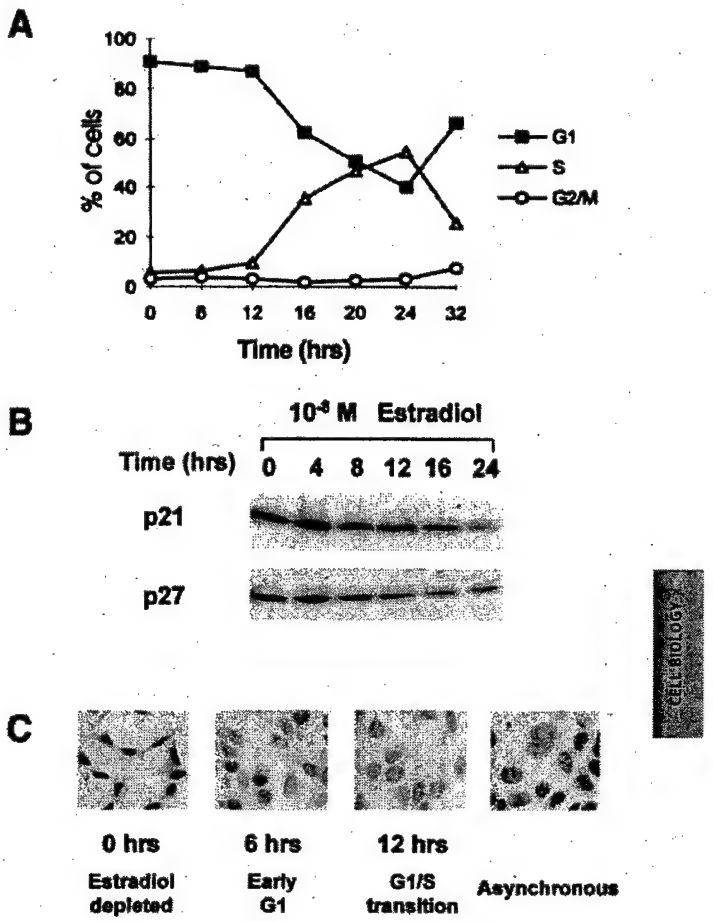

Fig. 1. Losses of p21 and p27 during estradiol stimulation of quiescent MCF-7 cells. Quiescent, estradiol-depleted MCF-7 cells were stimulated by readdition of 10 nM estradiol, and samples were taken at intervals thereafter. (*A*) Cell cycle synchrony was determined by dual BrdUrd/propidium iodide pulse labeling and flow cytometric analysis. (*B*) p21 and p27 immunoblots revealed levels of these proteins during cell cycle progression. (*C*) p27 protein was assayed by immunohistochemistry in asynchronous cultures at the indicated times after estradiol stimulation of quiescent MCF-7 as described (25).

p27 staining that was notably reduced by 6 h after addition of estradiol and barely detectable above background by 12 h as S phase entrance began.

The pattern of binding of p21 and p27 to cdk4 and cdk2 complexes differed during estradiol-stimulated cell cycle progression in MCF-7. Cdk4-bound p27 was abundant in estradiol-depleted cells and increased in parallel with the increased assembly and activation of cyclin D1–cdk4 between 3 and 9 h after estradiol addition (Fig. 2 *A* and *B*). Cyclin D1-dependent kinase activities and cyclin D1 binding to cdk4 were reduced by 12 h and undetectable by 16 h. Although p21 protein was elevated, very little p21 was detectable in cdk4 complexes in quiescent MCF-7. As for p27, cyclin D1–cdk4 assembly was accompanied by increased p21 binding, in keeping with the function of p21 and p27 as positive regulators of cyclin D1–cdk4 assembly (37, 41). Although cyclin D2 and cdk6 are detectable in MCF-7, cyclin D1–cdk4 has been shown to be the major D-type cdk in these cells (31, 32). In contrast to their pattern in cyclin D1 complexes, activation of cyclin E–cdk2 after estradiol stimulation was correlated with loss of p27 and p21 from cyclin E–cdk2. Cyclin E–cdk2 activation was correlated with S phase entrance (Fig. 2 *B* and *C*).

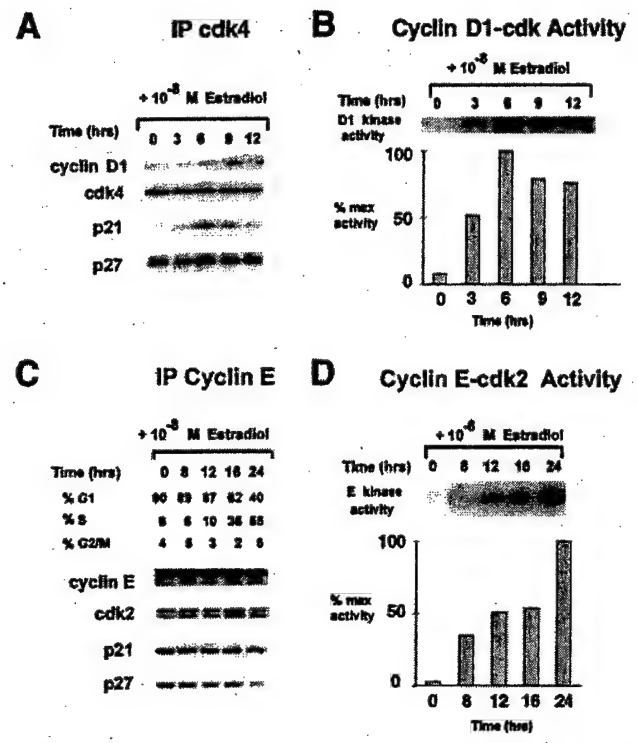

Fig. 2. Different patterns of KIP binding during cyclin D1–cdk4 and cyclin E–cdk2 activation. Cdk4 (*A*) and cyclin E (*C*) immunoprecipitates (IP) from cell lysates recovered at intervals after readdition of estradiol to steroid-depleted MCF-7 cells were resolved and analyzed by immunoblotting with the indicated antibodies. Cyclin D1 (*B*) and cyclin E (*D*) immunoprecipitates were assayed for kinase activity (36) at intervals after estradiol stimulation of quiescent MCF-7.

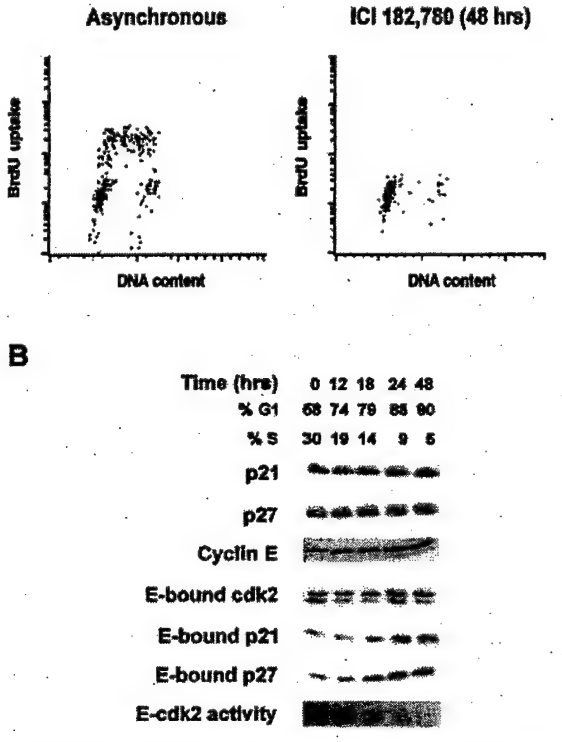

Fig. 3. p21 and p27 proteins increase during G_0/G_1 arrest by ER blockade. Asynchronously growing MCF-7 cells were treated with the ER-blocking drug ICI 182780 (Faslodex) at time 0 h, and samples were collected for flow cytometry or protein analysis at times indicated. (*A*) Cell cycle distribution before and 48 h after drug treatment. (*B*) Lysates were analyzed by immunoblotting with the indicated antibodies. Cyclin E immunoprecipitates were immunoblotted for associated p21 or p27 or analyzed for associated histone H1 kinase activity as described in ref. 36. Similar results were obtained for arrests with 4-OH TAM or after transfer to estradiol-depleted, charcoal-stripped serum.

p21 and p27 Bind and Inhibit Cyclin E–cdk2 on Interruption of Mitogenic ER Signaling. To investigate further cell cycle regulation by estrogen, ER signaling was interrupted in asynchronous MCF-7 cultures in three ways: by treatment with the pure ER antagonist ICI 182780, by the addition of antiestrogen 4-OH TAM, or by steroid depletion. All induced quiescence, with the S phase fraction falling from 29–36% to 1–5% over 48 h with a corresponding increase in the percentage of cells in G_0/G_1 phase (data shown for ICI 182780 in Fig. 3). p21 and p27 proteins increased, as did their binding to cyclin E–cdk2, in parallel with kinase inhibition (Fig. 3*B*). Although levels of cyclin E-bound cdk2 were unchanged, there was an accumulation of the slower-mobility, non-CAK-activated cdk2, lacking Thr-160 phosphorylation. The pattern of increase in p21 and p27 and of their binding to cyclin E during estradiol depletion and 4-OH TAM arrest were similar to that shown for ICI 182780 in Fig. 3*B*. The loss of cyclin D1 observed during antiestrogen treatment would lead to dissociation of KIPs from cyclin D1 complexes and foster KIP inhibition of cyclin E–cdk2 (42, 43).

These data and earlier work support the notion that estrogens and antiestrogens work through changes in p21 and p27 levels. Foster and Wimalasen (33) showed that p27 immunoprecipitation significantly depleted cyclin E–cdk2 inhibitory activity from serum and amino acid-starved MCF-7 cells. Others showed that most of the cyclin E–cdk2 inhibitory activity in Tamoxifen- or ICI 182780-arrested MCF-7 was removed by immunodepletion of p21. However, immunodepletion of both p21 and p27 was required to deplete fully cyclin E from arrested cells, indicating that cyclin E is bound to either p21 or p27 in an ER-blocked arrest state (31, 32, 42). These authors proposed that the estradiol-stimulated up-regulation of cyclin D1 served to sequester p21 away from cyclin E complexes leading to activation of cyclin E and pRb phosphorylation. However, none of these earlier studies definitively established a requirement for p21 and p27 in cell cycle arrest by antiestrogens.

p21 and p27 Are Essential Mediators of Arrest by Antiestrogens. To test directly whether p21 and p27 play essential roles in the cell cycle arrest after blockade of ER signaling in MCF-7 cells, we used antisense oligonucleotides to inhibit p21 (ASp21) or p27 (ASp27) expression in cells arrested by ICI 182780, 4-OH TAM, or estradiol depletion (data shown for ASp27 in estradiol-depleted MCF-7 in Fig. 4). A G clamp heterocycle modification, a cytosine analog that clamps onto a guanine, was designed to enhance antisense/RNA interaction and showed increased antisense oligonucleotide potency over the C5 propynyl-modified oligonucleotides used in earlier assays (38, 39). Within 1 h of transfection, ASp27-treated cells showed a 4-fold reduction in p27 but no loss of p21 (Fig. 4*A*), and p27 levels reached a nadir at about 6 h after transfection. The ASp21 oligos showed a similar specificity with no immediate loss of p27. Metabolic pulse labeling of ASp27-transfected cells showed specific inhibition of p27 synthesis but no effect on p21 protein synthesis at 1 and 21 h after completion of the transfection (Fig. 4*A*).

Treatment with ASp27 led to hyperphosphorylation of pRb and p130, CAK phosphorylation at Thr-160 on cdk2 (Fig. 4*B*), loss of p27 binding to cyclin E–cdk2, and cyclin E–cdk2 activation (Fig. 4 *C* and *D*), all consistent with stimulation of G_1-to-S phase progression. Similar findings were observed for ASp21-

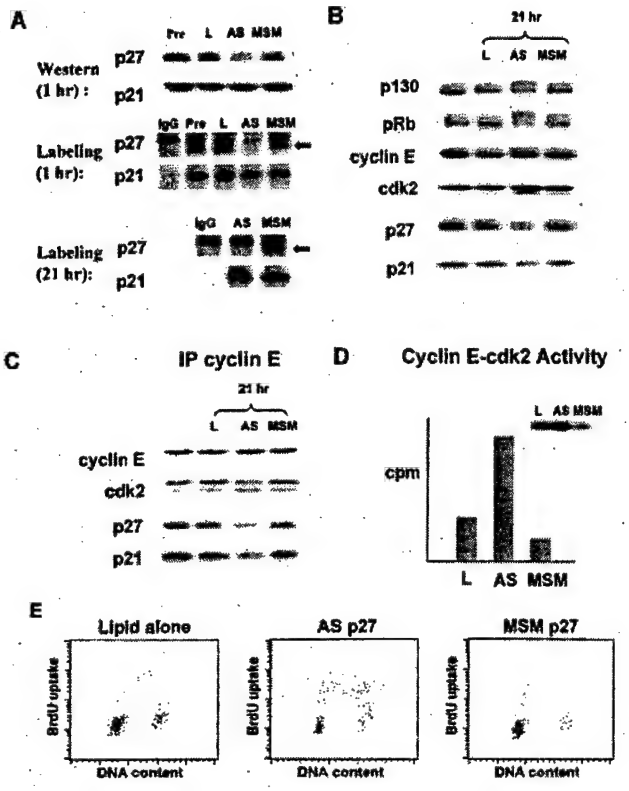

Fig. 4. Requirement of p27 for cell cycle arrest by estradiol depletion. (*A*) Estradiol-depleted MCF-7 cells were lysed before (left lane) or 1 h after exposure to lipid only (L), ASp27 (AS), or MSMp27 (MSM) and were immunoblotted for p21 and p27. Before and at 1 and 21 h after ASp27 transfection, cells were metabolically pulse labeled with [^{35}S]methionine, and p21 and p27 were immunoprecipitated from lysates containing equal trichloroacetic acid incorporation. The positions of metabolically labeled p27 are indicated by arrows. A nonspecific band, migrating close to p27 was present in all lanes, including the control nonspecific IgG lane. (*B*) Immunoblotting shows cell cycle regulatory protein levels before (left lane) or 21 h after transfection of lipid alone (L), ASp27, or MSMp27. Cyclin E immunoprecipitates (IP) were recovered from the same lysates as in *B* above and immunoblotted to detect associated proteins (*C*) or assayed for cyclin E-associated histone H1 kinase activity (*D*). (*E*) Flow cytometry 21 h after transfection with ASp27, lipid alone, or MSMp27.

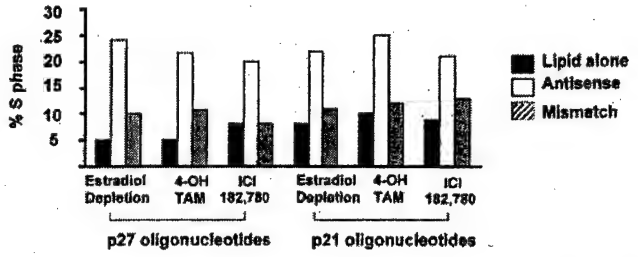

Fig. 5. Requirement for p21 and p27 in G_1 arrest by ER-blocking drugs or estradiol-depletion. MCF-7 cells were arrested by estradiol depletion or by treatment with 4-OH TAM or ICI 182780. The graph indicates the percentage of S phase cells after a 21-h exposure to lipid only (black bars), antisense (white bars), or mismatch (hatched bars) oligonucleotides to either p21 or p27.

treated cells. ASp21 treatment of cells arrested by estradiol-depletion, 4-OH TAM, or ICI 182780 showed loss of p21 and loss of p21 from cyclin E–cdk2 with activation of this kinase accompanying S phase entry (not shown). These effects were not observed in the mismatch and lipid control groups. Results shown are representative of up to three repeat experiments. It is notable that ASp27-treated cells showed late down-regulation of p21 at 21 h after transfection. Metabolic labeling of ASp27-transfected cells showed persistent specific inhibition of p27 synthesis at 21 h but no inhibition of p21 synthesis as cells were entering S phase. Thus, the reduction of p21 protein is not due to inhibition of p21 synthesis by the ASp27 oligo. Rather, the ASp27-induced inhibition of p27 synthesis was sufficient to move cells out of quiescence, and the subsequent loss of p21 most likely reflects the changes in posttranslational regulation of p21 leading to its degradation at the G_1-to-S phase transition (44). Data in Fig. 4 *A–C* for ASp27 treatment of ICI 182780-arrested cells were similar to results for ASp27 treatment of cells arrested by estradiol depletion or 4-OH TAM (not shown). Despite the continued blockade of ER signaling, ASp21 or ASp27 transfection stimulated cell cycle entry of cells arrested by steroid depletion, 4-OH TAM, or ICI 182780. The S phase fraction rose to 21–26% at 21 h (or 15 h after reaching minimum levels of the AS-targeted KIP protein, Figs. 4*E* and 5) and 29–36% at 28 h after transfection. Thus, ASp21 or ASp27 was sufficient to mimic the effect of estradiol on G_1-to-S phase progression in MCF-7 cells. These data indicate that a key effect of ER signaling is to relieve KIP-mediated inhibition of cdk2.

ASp27 not only inhibits p27 synthesis but would also lead to an increase in p27 degradation. As cyclin E–cdk2 is liberated from bound p27, it then phosphorylates remaining p27 molecules on Thr-187, thereby accelerating p27 degradation (45–47). Moreover, the activation of cyclin E–cdk2 is autocatalytic through activation of Cdc25A by cyclin E–cdk2 (48, 49). Thus, a relatively small initial reduction in p27 can stimulate a significant loss of p27 from cyclin E complexes.

Although breast cancer cells arrested by interruption of ER signaling have high levels of both p21 and p27, only p27 is elevated in serum-starved fibroblasts, and p27 is essential for arrest by serum starvation in immortalized fibroblasts (50, 51). Although p21 can compensate in part for the lack of p27 in serum-starved p27-null murine embryo fibroblasts (52), the mechanisms of quiescence induced by serum starvation in fibroblasts and those induced by steroid depletion in malignant breast epithelial cells differ importantly. Our data demonstrate that the cell cycle arrest induced by estradiol depletion or ER blockade requires both p21 and p27 and that these KIPs are not merely up-regulated as a consequence of cell cycle arrest.

Mitogenic effects of estradiol include the up-regulation of cyclin D1 through increased transcription (42, 43) and stabilization of cyclin D1 protein by assembly with cdk4 and cdk6 (53, 54), the latter mediated by p21 and p27 (37, 41). In addition, the present data establish definitively that estradiol-mediated losses of p21 and p27 relieve the inhibition of cyclin E–cdk2 (31, 32, 42).

In estradiol-depleted MCF-7, although p21 and p27 were abundant, they were not competent to stabilize cyclin D1 via assembly with its cdk partners. Cyclin D1 synthesis is detectable in metabolically labeled quiescent breast epithelial cells, but its association with cdk4 or cdk6 is detectable only several hours after mitogenic stimulation (ref. 36 and S. Cariou and J. Slingerland, unpublished results). Thus, an important effect of estradiol may be the conversion of p21 and p27 from a form that does not support assembly of cyclin D–cdk complexes in G_0, to one that does. Similarly, in serum-starved fibroblasts, p27 did not support cyclin D1–cdk4 complex formation even after ectopic cyclin D1 overexpression (55). Moreover, in p21/p27 null cells, overexpression of cyclin D1 did not permit its assembly with cdk4 (41). After estradiol stimulation in MCF-7, KIP-cyclin D1–cdk4 assembly occurred at 6–9 h, whereas the loss of p27 and p21 from cyclin E complexes was notable only somewhat later, after the time of peak sequestration of these KIPs in cyclin D1 complexes. Although induced overexpression of cyclin D1 can abrogate

antiestrogen arrest (42, 43), the physiologic up-regulation of cyclin D1 stimulated by estradiol in MCF-7 may be insufficient, on its own, to mediate the shifts of the KIPs out of cdk2.

The ubiquitin-mediated degradation of p27 (56–59) requires its phosphorylation on Thr-187 (45–47). Degradation of p21 is also proteasome dependent but may differ importantly from that of p27 (44). Although cyclin E–cdk2 acts *in vitro* and *in vivo* to phosphorylate p27 on Thr-187, other kinases may act on p27 before its degradation. The transition of p21 and p27 from potent inhibitors of cyclin E–cdk2 in G_0 to cyclin D-dependent kinase assembly factors may require phosphorylation early in G_1, before cyclin E–cdk2 activation. We have observed an increase in p27 phosphorylation before the reduction in its steady-state levels in estradiol-stimulated MCF-7 (S. Cariou and J. Slingerland, unpublished results). Mitogen-activated protein kinase activation has been implicated in p27 degradation (60, 61). It will be of interest to determine whether the estradiol-dependent activation of mitogen-activated protein kinase (16, 17) or other mitogenic kinase pathways regulate the transition of p21 and p27 from high-affinity inhibitors of cyclin E–cdk2, to activators of cyclin D1–cdk assembly.

The approximation that over 4 million women with breast cancer are on Tamoxifen worldwide is a minimal estimate (refs. 3 and 4 and V. C. Jordan, personal communication). An increasing body of *in vitro* data and metaanalysis of large patient cohorts have confirmed the requirement for ER expression for the therapeutic efficacy of Tamoxifen (3, 4). The present study suggests that, in addition to the ER, a breast cancer cell must express functional p21 and p27 for Tamoxifen or Faslodex (ICI 182780) to mediate cytostatic effects. These observations raise the hypothesis that deregulation and loss of these KIPs may underlie the clinical phenomena of hormone independence and antiestrogen resistance in breast cancer.

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1. Musgrove, E. A. & Sutherland, R. L. (1994) *Cancer Biol.* **5**, 381–389.
2. Henderson, B. E., Roos, R. & Bernstein, L. (1988) *Cancer Res.* **48**, 246–253.
3. Jordan, V. C. (1995) *Breast Cancer Res. Treat.* **36**, 267–285.
4. Early Breast Cancer Trialists' Collaborative Group (1998) *Lancet* **351**, 1451–1467.
5. Sutherland, R. L., Green, M. D., Hall, R. E., Reddel, R. R. & Taylor, I. W. (1983) *Eur. J. Cancer Clin. Oncol.* **19**, 615–621.
6. Osborne, C. K., Boldt, D. H., Clark, G. M. & Trent, J. M. (1983) *Cancer Res.* **43**, 3583–3585.
7. Watts, C. K. W., Brady, A., Sarcevic, B., deFazio, A. & Sutherland, R. L. (1996) *Mol. Endocrinol.* **9**, 1804–1813.
8. Nicholson, R. I., Francis, A. B., McClelland, R. A., Manning, D. L. & Gee, J. M. W. (1994) *Endocr. Relat. Cancer* **3**, 1–13.
9. Encarnacion, C. A., Ciocca, D. R., McGuire, W. L., Clark, G. M., Fuqua, S. A. & Osborne, C. K. (1993) *Breast Cancer Res. Treat.* **26**, 237–246.
10. Robertson, J. F. R. (1996) *Br. J. Cancer* **73**, 5–12.
11. Howell, A., DeFriend, D., Robertson, J., Blamey, R. & Walton, P. (1995) *Lancet* **245**, 29–30.
12. Howell, A., DeFriend, D. J., Robertson, J. F. R., Blamey, R. W., Anderson, L., Anderson, E., Sutcliffe, F. A. & Walton, P. (1996) *Br. J. Cancer* **74**, 300–308.
13. Perlmann, T. & Evans, R. M. (1997) *Cell* **90**, 391–397.
14. Beato, M., Chavez, S. & Truss, M. (1996) *Steroids* **61**, 240–251.
15. Katzenellenbogen, J. A., O'Malley, B. W. & Katzenellenbogen, B. S. (1996) *Mol. Endocrinol.* **10**, 119–131.
16. Migliaccio, A., DiDomenico, M., Castoria, C., DeFalco, A., Bontempo, P., Nola, E. & Auricchio, F. (1996) *EMBO J.* **15**, 1292–1300.
17. Collins, P. & Webb, C. (1999) *Nat. Med.* **5**, 1130–1131.
18. van der Kwast, T. H., Schalken, J., Ruizeveld de Winter, J. A., van Vroonhoven, C. C., Mulder, E., Boersma, W. & Trapman, J. (1991) *Int. J. Cancer* **48**, 189–193.
19. Ruizeveld de Winter, J. A., Janssen, P. J., Sleddens, H. M., Verleun-Mooijman, M. C., Trapman, J., Brinkmann, A. O., Santerse, A. B., Schroder, F. H. & van der Kwast, T. H. (1994) *Am. J. Pathol.* **144**, 735–746.
20. Solomon, M. J. & Kaldis, P. (1998) *Results Probl. Cell Differ.* **22**, 79–109.
21. Morgan, D. O. (1995) *Nature (London)* **374**, 131–134.
22. Sherr, C. J. (1994) *Cell* **79**, 551–555.
23. Reed, S. I., Bailly, E., Dulic, V., Hengst, L., Resnitzky, D. & Slingerland, J. (1994) *J. Cell Sci. Suppl.* **18**, 69–73.
24. Sherr, C. J. & Roberts, J. M. (1999) *Genes Dev.* **13**, 1501–1512.
25. Catzavelos, C., Bhattacharya, N., Ung, Y. C., Wilson, J. A., Roncari, L., Sandhu, C., Shaw, P., Yeger, H., Morava-Protzner, I., Kapusta, L., *et al.* (1997) *Nat. Med.* **3**, 227–230.
26. Tan, P., Cady, B., Wanner, M., Worland, P., Cukor, B., Magi-Galluzzi, C., Lavin, P., Draetta, G., Pagano, M. & Loda, M. (1997) *Cancer Res.* **57**, 1259–1263.
27. Wakasugi, E., Kobayashi, T., Tamaki, Y., Ito, Y., Miyashiro, I., Komoike, Y., Takeda, T., Shin, E., Takatsuka, Y., Kikkawa, N., *et al.* (1997) *Am. J. Clin. Pathol.* **107**, 684–691.
28. Jiang, M., Shao, Z.-M., Wu, J., Lu, J.-S., Yu, L.-M., Yuan, J.-D., Han, Q.-X., Shen, Z.-Z. & Fontana, J. A. (1997) *Int. J. Cancer* **74**, 529–534.
29. Tsihlias, J., Kapusta, L. & Slingerland, J. (1999) *Annu. Rev. Med.* **50**, 401–423.
30. Saez, A., Sanchez, E., Sanchez-Beato, M., Cruz, M. A., Chacon, I., Munoz, E., Camacho, F. I., Martinez-Montero, J. C., Mollejo, M., Garcia, J. F., *et al.* (1999) *Br. J. Cancer* **80**, 1427–1434.
31. Prall, O. W. J., Sarcevic, B., Musgrove, E. A., Watts, C. K. W. & Sutherland, R. L. (1997) *J. Biol. Chem.* **272**, 10882–10894.
32. Planas-Silva, M. D. & Weinberg, R. A. (1997) *Mol. Cell. Biol.* **17**, 4059–4069.
33. Foster, J. & Wimalasen, J. (1996) *Mol. Endocrinol.* **10**, 488–496.
34. Soule, H. D., Vazquez, J., Long, A., Albert, S. & Brenan, S. (1973) *J. Natl. Cancer Inst.* **51**, 1409–1413.
35. Petrocelli, T., Poon, R., Drucker, D., Slingerland, J. & Rosen, C. (1996) *Oncogene* **12**, 1387–1396.
36. Sandhu, C., Garbe, J., Daksis, J., Pan, C.-H., Bhattacharya, N., Yaswen, P., Koh, J., Slingerland, J. & Stampfer, M. R. (1997) *Mol. Cell. Biol.* **17**, 2458–2467.
37. LaBaer, J., Garrret, M., Steenson, M., Slingerland, J., Sandhu, C., Chou, H., Fattaey, A. & Harlow, H. (1997) *Genes Dev.* **11**, 847–862.
38. Flanagan, W. M., Wolf, J. J., Olson, P., Grant, D., Lin, K. Y., Wagner, R. W. & Matteucci, M. D. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 3513–3518.
39. St. Croix, B., Florenes, V., Rak, A., Flanagan, J. W., Bhattacharya, N., Slingerland, J. M. & Kerbel, R. S. (1996) *Nat. Med.* **2**, 1204–1210.
40. Musgrove, E., Lilischkis, R., Cornish, A. L., Lee, S. L., Setlur, V., Seshari, R. & Sutherland, R. L. (1995) *Int. J. Cancer* **63**, 584–591.
41. Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M. & Sherr, C. J. (1999) *EMBO J.* **18**, 1571–1583.
42. Watts, C. K., Sweeney, K. J. E., Warlters, A., Musgrove, E. A. & Sutherland, R. L. (1994) *Breast Cancer Res. Treat.* **31**, 95–105.
43. Prall, O. W. J., Rogan, E. M., Musgrove, E. A., Watts, C. K. W. & Sutherland, R. L. (1998) *Mol. Cell. Biol.* **18**, 4499–4508.
44. Sheaff, R. J., Singer, J. D., Swanger, J., Smitherman, M., Roberts, M. J. & Clurman, B. E. (2000) *Mol. Cell* **5**, 403–410.
45. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M. & Clurman, B. E. (1997) *Genes Dev.* **11**, 1464–1478.
46. Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A. & Pagano, M. (1999) *Genes Dev.* **13**, 1181–1189.
47. Vlach, J., Hennecke, S. & Amati, B. (1997) *EMBO J.* **16**, 5334–5344.
48. Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H. & Okayama, H. (1994) *EMBO J.* **13**, 1549–1556.
49. Hoffmann, I., Draetta, G. & Karsenti, E. (1994) *EMBO J.* **13**, 4302–4310.
50. Rivard, N., L'Allemain, G., Bartek, J. & Pouyssegur, J. (1996) *J. Biol. Chem.* **271**, 18337–18341.
51. Coats, S., Flanagan, M., Nourse, J. & Roberts, J. M. (1996) *Science* **272**, 877–880.
52. Coats, S., White, P., Fero, M. L., Lacy, S., Chung, G., Randel, E., Firpo, E. & Roberts, J. M. (1999) *Curr. Biol.* **9**, 163–173.
53. Bates, S., Parry, D., Bonetta, L., Vousden, K., Dickson, C. & Peters, G. (1994) *Oncogene* **9**, 1633–1640.
54. Parry, D., Bates, S., Mann, D. J. & Peters, G. (1995) *EMBO J.* **14**, 503–511.
55. Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. A. & Kato, J.-Y. (1994) *Mol. Cell. Biol.* **14**, 2066–2076.
56. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F. & Rolfe, M. (1995) *Science* **269**, 682–685.
57. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H. & Zhang, H. (1999) *Curr. Biol.* **9**, 661–664.
58. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U. & Krek, W. (1999) *Nat. Cell Biol.* **1**, 207–214.
59. Carrano, A., Eytan, E., Hershko, A. & Pagano, M. (1999) *Nat. Cell Biol.* **1**, 193–199.
60. Alessandrini, A., Chiaur, D. S., Erikson, R. & Pagano, M. (1997) *Leukemia* **11**, 342–345.
61. Kawada, M., Yamagoe, S., Murakami, Y., Suzuki, K., Mizuno, S. & Uehara, Y. (1997) *Oncogene* **15**, 629–637.

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Constitutive MEK/MAPK Activation Leads to $p27^{Kip1}$ Deregulation and Antiestrogen Resistance in Human Breast Cancer Cells*

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Antiestrogens, such as the drug tamoxifen, inhibit breast cancer growth by inducing cell cycle arrest. Antiestrogens require action of the cell cycle inhibitor $p27^{Kip1}$ to mediate G_1 arrest in estrogen receptor-positive breast cancer cells. We report that constitutive activation of the mitogen-activated protein kinase (MAPK) pathway alters p27 phosphorylation, reduces p27 protein levels, reduces the cdk2 inhibitory activity of the remaining p27, and contributes to antiestrogen resistance. In two antiestrogen-resistant cell lines that showed increased MAPK activation, inhibition of the MAPK kinase (MEK) by addition of U0126 changed p27 phosphorylation and restored p27 inhibitory function and sensitivity to antiestrogens. Using antisense p27 oligonucleotides, we demonstrated that this restoration of antiestrogen-mediated cell cycle arrest required p27 function. These data suggest that oncogene-mediated MAPK activation, frequently observed in human breast cancers, contributes to antiestrogen resistance through p27 deregulation.

$p27^{kip1}$ is a member of the KIP[1] (kinase inhibitory protein) family of cdk inhibitors that regulate the cyclin-cdk complexes governing cell cycle transitions (1). The importance of p27 as a G_1-to-S phase regulator is highlighted by the finding that antisense-mediated inhibition of p27 expression is sufficient to induce cell cycle entry in quiescent fibroblasts (2) and in steroid-depleted breast cancer cells (3). p27 protein levels are high in G_0 and early G_1 during which time p27 binds tightly and inhibits cyclin E1-cdk2. p27 translation rates decrease, and its proteolysis increases during G_1-to-S phase progression, leading to p27 protein loss as cells enter S phase (4–6). p27 proteolysis is regulated by phosphorylation of p27 on threonine 187 (Thr-187) by cyclin E1-cdk2 (7, 8). While mutations or deletions in the p27 gene are uncommon (9, 10), p27 degradation is increased in many cancers, including breast cancer (11, 12).

An increasing body of data suggests that p27 is regulated by mitogenic signal transduction pathways, including Ras-dependent activation of the mitogen-activated protein kinase (MAPK) pathway (13–17). Many mitogens increase the cellular levels of GTP-bound Ras, leading to activation of the downstream target, Raf-1. The Raf-1 kinase can phosphorylate and activate the dual specificity kinases MEK1 and MEK2, which in turn activate MAPK (also known as $p42^{ERK2}$ and $p44^{ERK1}$). Once activated, MAPK can phosphorylate several nuclear transcription factors including Myc, Elk, and Rsk (for review, see Ref. 18). p27 itself has several MAPK consensus sites, and MAPK can phosphorylate p27 *in vitro* (16) and reduce the ability of recombinant p27 to bind and inhibit cdk2 *in vitro* (15). While constitutive activation of Ras-MAPK can reduce p27 inhibitory function in immortal and cancer-derived lines, it is not clear whether MAPK directly regulates p27 during cell cycle progression in normal cell types.

Studies of p27 regulation by the Ras-MAPK pathway were initially carried out in fibroblasts (15, 19, 20). In NIH3T3 fibroblasts, Ras signaling is required for the down-regulation of p27 as cells approach the G_1-to-S phase transition (13, 20). Introduction of a dominant negative *ras* mutant prevented the loss of p27 in response to serum and inhibited S phase entry. Others have reported that Ras-MAPK activation reduces the ability of p27 to inhibit cdk2 through sequestration of p27 into cyclin D1-cdk4 complexes, rather than by promoting p27 protein loss (14).

Constitutive activation of the MAPK cascade may contribute to malignant progression of many human cancers (21). Although the causes of MAPK activation differ among tumors, in many cancers constitutive signaling from cell surface tyrosine kinase receptors contributes to activation of the Ras-Raf-1-MEK-MAPK pathway. For example, the epidermal growth factor receptor and HER2/c-ErbB-2, both of which activate the Ras-MAPK pathway, are overexpressed in up to 20 and 30% of breast cancers, respectively. Overexpression of these receptors has been associated with antiestrogen resistance and poor prognosis in primary breast cancers (22–27). Tissue culture models suggest that elevated MAPK activity may contribute to estrogen-independent growth of breast cancer cells (28–30).

Antiestrogen drugs, such as tamoxifen, are effective in the treatment and prevention of breast cancer (31–33). However, only two-thirds of estrogen receptor (ER)-positive breast cancers respond initially to antiestrogen therapy, and even sensitive tumors invariably acquire antiestrogen resistance (34). In most cases, acquired resistance is not due to a loss or mutation of the ER (35, 36). Numerous mechanisms have been proposed to explain the phenomenon of tamoxifen-resistant ER-positive breast cancer, including altered drug metabolism (37), altered binding of co-activator and co-repressor molecules to the antiestrogen-ER complex (38), and altered signal transduction

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[1] The abbreviations used are: KIP, kinase inhibitory protein; cdk, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ER, estrogen receptor; BrdUrd, bromodeoxyuridine; 2D-IEF, two-dimensional isoelectric focusing; AS, antisense.

pathways that modulate ER activity (39) or regulate the cell cycle machinery (3).

The cell cycle inhibitor, $p27^{Kip1}$, is an essential mediator of cell cycle arrest by tamoxifen and other antiestrogenic drugs. We recently demonstrated that antisense-mediated down-regulation of $p27^{kip1}$ abrogated antiestrogen-induced cell cycle arrest in the ER-positive MCF-7 breast cancer line (3). p27 protein levels are frequently reduced in primary breast cancers compared with the normal breast epithelium, and low p27 protein levels are associated with poor prognosis and hormone independence (11, 40, 41). These observations stimulated the present study to investigate the relationships between Ras-MAPK pathway activation, antiestrogen resistance, and p27 function. Our data indicate that constitutive MEK activation alters p27 phosphorylation, reduces p27 inhibitory activity, and contributes to antiestrogen resistance in breast cancer.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7 cells (42) and LY-2 cells (43) were obtained from the laboratory of M. Lippman. The LCC2 line was a gift from G. Clarke (44). MCF-7 cells, stably transfected with full-length *HER2* cDNA (MCF-7/HER2–18), were kindly provided by C. Arteaga. All lines were grown in improved-modified essential medium (option Zn^{2+}) supplemented with insulin and 10% fetal calf serum.

Flow Cytometric Analysis—Cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdUrd) for 2 h and then fixed, stained with anti-BrdUrd-conjugated fluorescein isothiocyanate (Becton Dickinson) and counterstained with propidium iodide as described (45). Cell cycle analysis was carried out on a Becton Dickinson FACScan and Cell Quest Software. Values given for flow cytometric analysis represent the mean of repeat assays.

Cell Cycle Effects of Antiestrogens and MEK Inhibition—For comparison of the effects of antiestrogens in MCF-7, MCF-7 transfectants, or LY-2 cell lines, cultures were treated by addition of 1 μM 4-hydroxy-tamoxifen (4-OH-TAM) (Sigma) or 10 nM ICI 182780 (7α-(9-(4,4,5,5,5-pentafluoropentylsulfinyl) nonyl)estra-1,3,5, (10)-triene-3,17β-diol, from Zeneca Pharmaceuticals) to complete medium, and samples were collected at 48 h thereafter for protein and flow cytometric analysis. The effects of MEK inhibition on the cell cycle were assayed following addition of 0.1 μM U0126 (Promega) for 2 or 24 h prior to recovery for immunoblotting or flow cytometric analysis. The effects of MEK inhibition on antiestrogen sensitivity in the antiestrogen-resistant lines, LY-2 or MCF-7/HER2–18, were assayed by treating cells with 0.1 μM U0126 for 2 h followed by an additional 48 h with either 1 μM 4-OH-TAM or 10 nM ICI 182780 prior to recovery of cells for protein or flow cytometric analysis.

Antisense Oligonucleotide Transfections—Antisense experiments were carried out as described (3). Phosphorothioate oligonucleotide sequences were as follows: GS5422 antisense p27 (ASp27) 5′-TG-GCTCTCXTGCGCC-3′; GS5585 mismatch p27 (MSMp27) 5′-TG-GCTCXCTTGCGCC-3′; *X* indicates the G-clamp modification of these oligonucleotides. The specificity of these oligonucleotides for p27 has been documented (2, 3). LY-2 cells were rendered quiescent by the addition of 0.1 μM U0126 (Promega) for 2 h followed by antiestrogen treatment (10 nM ICI 182780, or 1 μM 4-OH TAM) for a further 48 h. Quiescent cells were then transfected with 120 nM oligonucleotides using 2.5 μg/ml cytofectin G3815 (Gilead Scientific, Foster City, California) for 6 h, followed by replacement with complete medium supplemented by U0126 and antiestrogen. Flow cytometry and proteins were analyzed prior to transfection and at 21 h thereafter.

Immunoblotting—Cells lysis and immunoblotting were as described (45). Equal protein loading was verified by blotting for β-actin. To assay cyclin E1 complexes, cyclin E1 was immunoprecipitated from 200 μg of protein lysate. Immunoprecipitates were resolved, transferred, and blotted with cyclin E1, cdk2, and p27 antibodies. Antibody alone controls were run along side immunoprecipitates.

Antibodies—Monoclonal antibodies to p27 were obtained from Neomarkers (DCS-72) or Transduction Laboratories. p27 rabbit polyclonal serum (pAb5588) was provided by H. Toyoshima and T. Hunter (Salk Institute). Antibodies to p21 were from Santa Cruz, to cdk2 (PSTAIRE) from S. Reed (The Scripps Research Institute), to MEK, MAPK, and phospho-MAPK from New England Biolabs, to β-actin from Sigma, and to cyclin E1 (mAbs E12 and E172) from E. Harlow (Massachusetts General). These cyclin E1 antibodies are specific for cyclin E1(46). The ER antibody H222 was provided by Dr. G. Greene.

Cyclin-dependent Kinase Assays—Cyclin E1 or p27 complexes were immunoprecipitated from 100 μg of protein lysate and reacted with [γ-^{32}P]ATP and histone H1 as described(45). Radioactivity incorporated in histone substrate was quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software. To determine background IgG-associated activity, nonspecific mouse IgG (for cyclin E1-cdk2 assays) or polyclonal rabbit IgG (for p27-associated kinase) immunoprecipitates from test lysates were collected on protein A, washed, and reacted with the kinase mixture as for cyclin E1 and p27 immunoprecipitates. Radioactivity incorporated in control nonspecific IgG immunoprecipitates was subtracted from test kinase values prior to graphing as in Refs. 45 and 47.

Production of Cyclin E1-cdk2 by Baculovirus Infection of Sf-9 Cells—Sf-9 cells and TNM-FH media were obtained from Invitrogen. Adherent Sf-9 cells were co-infected with baculoviruses encoding human *cyclin E1* or human *cdk2* genes, and cyclin E1 and cdk2 were prepared as described (46). Sf-9 cell lysates containing cyclin E1 and cdk2 were used directly in p27 inhibitor assays.

Assays of p27 Inhibitory Function—Cell lysates (250 μg) from asynchronously proliferating MCF-7, MCF-7/MEK^{EE} (line M2), or LY-2 cells were immunoprecipitated with pAb5588 anti-p27 serum or control polyclonal rabbit IgG, and precipitates were collected on protein A-Sepharose beads. For testing heat-stable p27 inhibitor activity, p27 immunoprecipitates were washed, then boiled for 5 min in 200 μl of reaction buffer, placed on ice, and then cleared by centrifugation. The p27 in the supernatant was recovered, and recombinant cyclin E1-cdk2 and dithiothreitol (1 mM final) were added and incubated at 30 °C for 30 min, followed by immunoprecipitation with either anti-cyclin E1 (mAbE172) or control polyclonal mouse IgG (Sigma). The un-inhibited control recombinant cyclin E1-cdk2 was treated with reaction buffer and incubated at 30 °C for 30 min without any added p27. Complexes were subsequently assayed for H1 kinase activity, and resulting activities were graphed as a % maximum un-inhibited cyclin E1-cdk2 activity.

Two-dimensional Isoelectric Focusing (2D-IEF) and Phosphatase Treatment—Cells were lysed in ice-cold 0.1% Tween 20 lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 2.5 mM EGTA, pH 8.0, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na_2VO_4, 0.5 mM dithiothreitol, and 0.02 mg/ml each of aprotinin, leupeptin, and pepstatin). For 2D-IEF, p27 immunoprecipitates were denatured in 8 M urea, loaded onto immobilized non-linear pH gradient (pH 3–10) IEF strips and focused at 50,000 volt-hour using the IPGphor IEF system (Amersham Pharmacia Biotech). These assays yield highly reproducible electrophoretic resolution of isoforms because of the covalent linkage of the electrophoresis medium to a matrix. The IEF strip was equilibrated in 50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS for 30 min before loading for SDS-polyacrylamide gel electrophoresis. Gels were transferred to polyvinylidene difluoride membrane, p27 isoforms were detected by immunoblotting using p27 antibody from Transduction Labs, and proteins were detected using enhanced chemiluminescence (ECL). For 2D-IEF of cyclin E1-bound p27, 3 mg of protein lysate was immunoprecipitated with the monoclonal mAb E172 antibody. Densitometric analysis of multiple ECL film exposures from repeat assays was used to determine the ratios of the various p27 isoforms. For phosphatase treatment, p27 immunoprecipitates were washed twice with phosphatase buffer (50 mM Tris, pH 8.0, 10% glycerol) and then incubated at 37 °C overnight with 66 units per 10 μl of reaction of calf intestinal alkaline phosphatase (Roche Molecular Biochemicals). To confirm that the most positively charged p27 isoforms represented unphosphorylated p27, cells were labeled with [^{32}P]orthophosphate (1 mCi/100-dish) for 3 h, and p27 immunoprecipitates were isolated and subjected to 2D-IEF. p27 immunoprecipitated from unlabeled cells was resolved by 2D-IEF in parallel with the labeled p27, and the resolution pattern of the cold p27 was compared with the phosphate-labeled p27.

RESULTS

p27 Deregulation in Antiestrogen-resistant LY-2—We compared p27 levels in antiestrogen-sensitive MCF-7 (42) and the resistant MCF-7 derivative, LY-2 (43). The loss of antiestrogen responsiveness in LY-2 was not due to a loss of p27 protein (Fig. 1*A*).

The association of p27 with cyclin E1-cdk2 was assayed in asynchronously growing and antiestrogen-treated MCF-7 and LY-2 cells (Fig. 1*B*). Asynchronously proliferating LY-2 and MCF-7 cells had similar cell cycle profiles (Fig. 1*B*). When equal amounts of cyclin E1 were immunoprecipitated, the

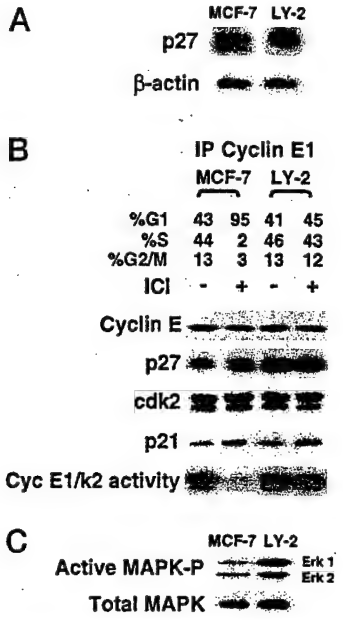

FIG. 1. **Antiestrogen-resistant LY-2 show altered p27 regulation.** *A*, asynchronously growing MCF-7 and LY-2 cell lysates were analyzed by Western blotting using the antibodies indicated. *B*, cyclin E1 immunoprecipitates (*IP*) from asynchronously proliferating and ICI 182780 (ICI)-treated cells were resolved and assayed for associated p27, p21, and cdk2 by immunoblotting or analyzed for associated histone H1 kinase activity as described under "Experimental Procedures." The cell cycle profiles from flow cytometric analysis are shown. *C*, lysates from asynchronously proliferating cells were analyzed for levels of total and active phospho-MAPK.

amount of p27 bound to cyclin E1-cdk2 in asynchronously growing LY-2 was nearly 4-fold higher than that in asynchronous MCF-7. There was no compensatory decrease in p21 binding to cyclin E1 in proliferating LY-2. Levels of cyclin E1-bound p21 were similar in proliferating MCF-7 and LY-2. Cyclin E1-bound cdk2 levels were similar in the two lines and were not affected by antiestrogens. Despite the increased p27 bound to cyclin E1-cdk2 in proliferating LY-2 cells, the histone H1 activity of these complexes was not reduced compared with cyclin E1-cdk2 from asynchronous MCF-7 (Fig. 1*B*). Antiestrogen treatment of MCF-7 with either ICI 182780 (ICI) or 4-OH-TAM (data shown here for ICI) caused a 3- to 5-fold increase in p27 binding to cyclin E1-cdk2, a 3-fold increase in cyclin E1-bound p21, inhibition of this kinase, and G_1 cell cycle arrest. Antiestrogen treatment of LY-2 caused a minimal increase in p21 binding, no change in the amount of p27 bound to cyclin E1-cdk2, no significant inhibition of cyclin E1-cdk2 activity, and no change in the cell cycle profile (Fig. 1*B*). These data suggested a functional alteration of p27 in LY-2 cells.

Since MAPK had been shown to alter p27 function in fibroblasts (15, 19, 20), we assayed MAPK activity by Western blotting using phospho-specific antibodies that detect activated MAPK. Although total MAPK protein levels were similar in LY-2 and MCF-7 cells, the levels of phosphorylated MAPK (both p42 ERK2 and p44 ERK1) were elevated nearly 8-fold in LY-2 (Fig. 1*C*). We also observed elevated MAPK activity in two other MCF-7 derived antiestrogen-resistant cell lines, LCC2 and MCF-7/HER2–18 (data not shown).

MEK1-transfected MCF-7 Lines Show Antiestrogen Resistance and Altered Binding of p27 to Cyclin E1-cdk2—To determine whether the increased MAPK activity observed in the antiestrogen-resistant lines was causally linked to the antiestrogen-resistant phenotype, we transfected MCF-7 cells with activated *MEK1* (MEK^{EE}) or an active *ERK2* allele (*ERK2-MEK*) and assayed stable transfected cell lines for antiestrogen sensitivity. Transfectants showed MAPK activation compared with parental cells and empty vector controls (representative data shown for MEK^{EE} transfectants, labeled M1 and M2 in Fig. 2*A*). Of note, both p21 and p27 levels were reduced in MEK^{EE} transfectants (Fig. 2*A*). The level of the ER protein was not affected by the degree of MAPK activation achieved in these cells (data not shown). Asynchronously growing MAPK-activated lines and empty vector controls showed similar cell cycle profiles (Fig. 2*B*). Lines with constitutive MAPK activation showed partial resistance to 4-OH-TAM or ICI compared with the parental or vector alone controls (Fig. 2*B*).

Levels of cyclin E1-associated p21 and p27 were assayed in asynchronous MEK^{EE} transfected lines and in the empty vector controls (Fig. 2*C*). Although densitometric analysis showed that total p27 levels in asynchronously growing MEK^{EE} transfectants were reduced by up to 3-fold compared with controls, the amount of p27 detected in cyclin E1-cdk2 complexes was not reduced (Fig. 2, *C* and *D*). Despite the similar amounts of both p27 and p21 bound to cyclin E1 in proliferating MEK^{EE} and control lines, equal amounts of cyclin E1-cdk2 showed approximately 2-fold higher kinase activity in MEK^{EE} transfectants compared with empty vector controls (Fig. 2, *C and D*). There was no change in cdk2 binding to cyclin E1, and the subtle increase in the proportion of the faster mobility, CAK-activated cdk2 bound to cyclin E1 would not suffice to mediate the 2-fold increase in cyclin E1-cdk2 activity in the MEK^{EE} lines (Fig. 2*C*). MCF-7 lines with constitutive MEK1 activation showed no increase in p27 binding to cyclin E1 following antiestrogens compared with that in parental MCF-7 or in the empty vector controls (representative data in Fig. 2*D*). The modest increase of p21 binding to cyclin E1-cdk2 may mediate the partial cell cycle inhibition after antiestrogen treatment of the M2 clone. Earlier work has established that increased KIP binding to cyclin E1-cdk2 in MCF-7 is essential for G_1 arrest by antiestrogens (3, 48). Thus, MAPK activation via MEK^{EE} or *ERK2-MEK* transfection may contribute to antiestrogen resistance, at least in part, by impairing the antiestrogen-mediated increase in p27 binding to cyclin E1-cdk2.

Altered p27 Phosphorylation in Antiestrogen-resistant Lines—The MAPK-activated transfectants and LY-2 cells show a number of similarities. Both showed more abundant p27 binding to cyclin E1-cdk2 in asynchronously proliferating cells than would have been predicted from the respective total cellular abundance of p27, and antiestrogens failed to cause an accumulation of p27 in cyclin E1-cdk2 complexes. We postulated that differences in p27 phosphorylation may be associated with these differences in p27 function. Under most one-dimensional SDS-polyacrylamide gel electrophoresis conditions, p27 does not show reproducible differences in gel mobility. 2D-IEF allowed resolution of different p27 phospho-isoforms that are not apparent on single dimension gel electrophoresis. 2D-IEF showed a reproducible difference between the phosphorylation profile of p27 in the antiestrogen-sensitive and -resistant lines. 2D-IEF of p27, using an amphoteric carrier with a non-linear pH range of 3–10, showed five p27 isoforms present in all three lines (labeled 1–5 in Fig. 2*E*). Form 1 migrates at the predicted isoelectric focusing point for p27 (pH = 6.54). Phosphatase treatment of the p27 immunoprecipitates confirmed that most of these different isoforms represent different phosphoforms of p27 (Fig. 2*E*). The minor amount of form 2 remaining after phosphatase treatment may reflect incomplete dephosphorylation. Alternatively, this may represent a hypophosphorylated form of p27 in which post-translational modification (*e.g.* myristylation) confers a more negative charge. When cells were [^{32}P]orthophosphate-labeled prior to p27 immunoprecipita-

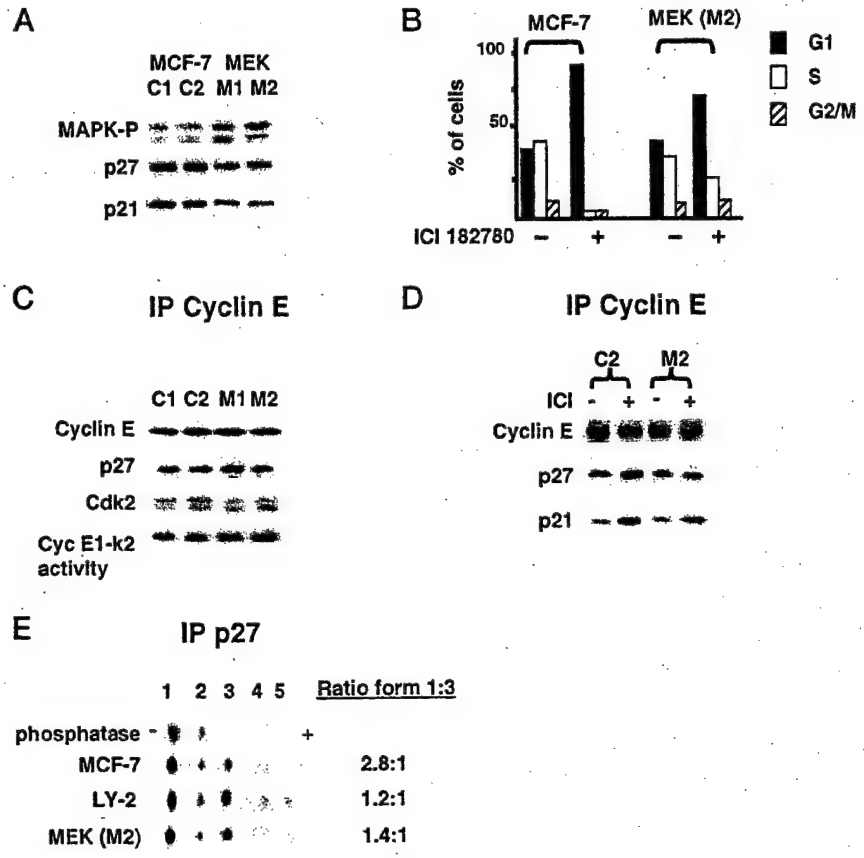

FIG. 2. **MAPK activation contributes to p27 deregulation and antiestrogen resistance.** *A*, the levels of active phospho-MAPK, p27, and p21 were analyzed in two control cell lines transfected with empty vector (C1, C2) and in two MEK overexpressing MCF-7 clones (M1, M2). *B*, the cell cycle profiles of asynchronously proliferating and antiestrogen-treated MEK transfectants were compared with empty vector controls. *C*, cyclin E1-bound p27 and cdk2 and cyclin E1-associated kinase activities were assayed as in Fig. 1*B*. *D*, cyclin E1-bound p21 and p27 were assayed before (−) and after (+) ICI treatment. *E*, p27 immunoprecipitates from asynchronously proliferating MCF-7, LY-2, and MEK^{EE}-transfectant and (M2), were analyzed by 2D-IEF. The 2D-IEF of phosphatase-treated p27 from MCF-7 cells is shown in the *upper panel*. The different p27 isoforms were quantitated by densitometry.

tion and resolution by 2D-IEF, isoforms 1 and 2 were not radiolabeled, confirming their hypophosphorylated state (data not shown).

The relative abundance of the different isoforms of p27 differed between MCF-7 and the MAPK-activated lines, LY-2 and MCF-7/MEK^{EE}. In MCF-7, most of the p27 focused as isoform 1 with a lesser amount as isoform 3, and the ratio of these two forms (isoform 1:isoform 3) quantitated by densitometry was 3:1. The 2D-IEF patterns of p27 from LY-2 and the MEK transfectants were similar, and both differed from that seen in MCF-7. In these antiestrogen-resistant lines, form 3 showed greater relative abundance and the ratios of form 1 to form 3 were similar (~1:1 in MEK^{EE} clone M2 and LY-2, Fig. 2*E*). These observations support the notion that MEK/MAPK activation modulates p27 phosphorylation in these resistant cell lines.

MEK Inhibition Restores Sensitivity to Antiestrogen-mediated Cell Cycle Arrest—Treatment of the LY-2 line with 0.1 μM of the MEK inhibitor, U0126, caused a 2.5-fold reduction of phospho-MAPK levels without affecting the total MAPK protein levels (Fig. 3*A*). Although this low dose of U0126 alone did not affect the cell cycle profile of the LY-2 cells, treatment with the combination of 0.1 μM U0126 and either 1 μM 4-OH-TAM or 10 nM ICI led to a G_1 arrest (data shown for ICI treatment, Fig. 3, *B and C*). MEK inhibition by 0.1 μM U0126 also restored 4-OH-TAM or ICI-mediated G_1 arrest in the antiestrogen-resistant HER2/ErbB-2 overexpressing line, MCF-7/HER2–18 (Fig. 3*B*). The G_1 arrest following the combination of MEK inhibition and antiestrogen treatment in LY-2 (U+ICI) was accompanied by a 5-fold increase in the binding of p27 to cyclin E1-cdk2 complexes (Fig. 3*C*) and inhibition of cyclin E1-cdk2 activity (data not shown). p21 binding to cyclin E1-cdk2 was also modestly increased, and the proportion of CAK-phosphorylated cdk2 (faster mobility) bound to cyclin E1 was modestly reduced by the combined ICI 182780 and U0126 treatment.

The Arrest of LY-2 by MEK Inhibition and Antiestrogens Is p27-dependent—The increase in p27 association with cyclin E1-cdk2 in LY-2 cells treated by the combination of 0.1 μM U0126 and 10 nM ICI was similar to that seen following antiestrogen treatment in the sensitive MCF-7 line (see Figs. 1*B* and 3*C*). We postulated that MAPK inhibition in LY-2 enhanced the cdk2 inhibitory function of p27 to facilitate cell cycle arrest by antiestrogens. If this were the case, then antisense-mediated inhibition of p27 expression in the U0126/ICI treated cells should abrogate this drug-mediated arrest. U0126/ICI-arrested LY-2 cells were transfected with either antisense p27 (ASp27) oligonucleotides or mismatch control oligonucleotides (MSp27) or mock transfected with lipid only (control, C), and cells were recovered for flow cytometry and protein analysis at 21 h following completion of ASp27 transfection. The inhibition of p27 expression in ASp27-treated cells lead to cell cycle re-entry with ~23% cells in S phase at 21 h, in contrast to 8 and 9% of cells in S phase following lipid only (control, C, or mismatch (MSp27) transfection (Fig. 3*D*). The ASp27-mediated cell cycle re-entry was associated with loss of cyclin E1-bound p27 and cyclin E1-associated kinase activation (Fig. 3*D*). Control (lipid alone) and MSp27-transfected groups showed no cyclin E1-cdk2 activation. We also observed a similar result using the combination of 0.1 μM U0126 and 1 μM 4-OH-TAM (data not shown). Thus, in the LY-2 line, p27 became an essential mediator of G_1 arrest by antiestrogens following partial MEK/MAPK inhibition.

p27-immunoprecipitable Kinase Activity in Antiestrogen-treated LY-2 Cells—Proliferating LY-2 cells, with and without antiestrogen treatment, showed more abundant p27 association with active cyclin E1-cdk2 than was detected in inhibited cyclin E1-cdk2 complexes from antiestrogen-arrested MCF-7 cells (Fig. 1*B*). These data suggested impaired inhibitory function of cyclin E1-bound p27 in LY-2 cells. p27 immunoprecipitates were tested for associated histone H1 kinase activity in

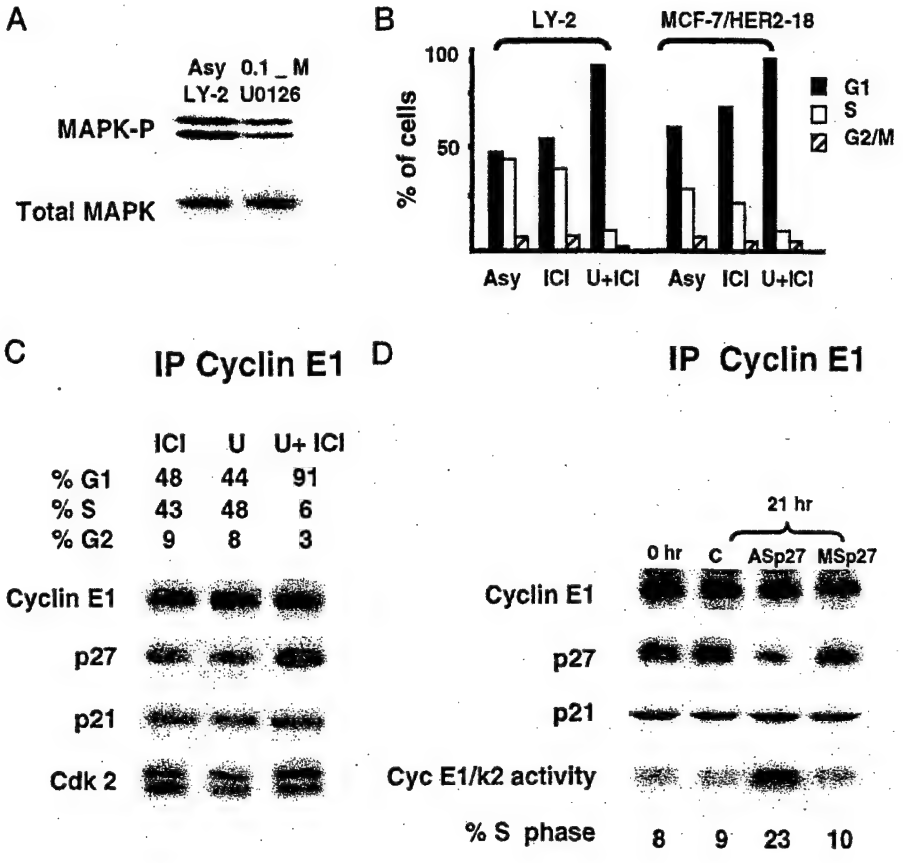

FIG. 3. **Inhibition of MAPK restores sensitivity to antiestrogens in LY-2.** *A*, asynchronously growing LY-2 or LY-2 cells treated with 0.1 μM U0126 were assayed for levels of total and active MAPK by immunoblotting. *B*, the cell cycle profile of LY-2 and MCF-7/HER2 cells were measured in asynchronously proliferating, ICI-treated and 0.1 μM U0126 + ICI (U+ICI)-treated cells. *C*, LY-2 cells treated for 48 h with ICI, U0126 (*U*) or the combination of U0126 + ICI (*U+ICI*) and cell cycle profiles assayed by flow cytometry. Cyclin E1 immunoprecipitates were resolved and analyzed by immunoblotting with the indicated antibodies. *D*, LY-2 cells treated with the combination of U0126 + ICI were lysed before (0 h) or 21 h after transfection with lipid only (control group, C), ASp27 oligonucleotides (ASp27), or MSp27 oligonucleotides (MSp27). Cyclin E1 immunoprecipitates were resolved and associated proteins detected by immunoblotting. The % S phase cells and cyclin E1-associated kinase activities in each treatment group are shown.

LY-2 and MCF-7 cells. Cdk2 complexes, but not cdk4 and 6 complexes, can use histone H1 as substrate. Histone H1 kinase activity was detected in p27 immunoprecipitates from asynchronous and ICI-treated LY-2 cells, but was negligible in asynchronous and ICI-treated MCF-7 when background activity in nonspecific antibody control immunoprecipitates was subtracted (Fig. 4*A*). The combination of 0.1 μM U0126 and 10 nM ICI 182780 (U+ICI) inhibited the p27 immunoprecipitable kinase activity in LY-2 (Fig. 4*A*). Parallel p27 immunoprecipitates were resolved and blotted for associated cdk2, cyclin E1, and cyclin A. The amounts of cyclin and cdk2 bound to p27 in ICI-treated LY-2 and MCF-7 were similar, and there was no loss of p27-bound cyclin or cdk2 following ICI plus U0126 treatment LY-2 cells (data not shown).

MEK Activation Modulates p27 Inhibitory Function—The inhibitory activity of p27 toward recombinant cyclin E1-cdk2 was compared in the MCF-7 and LY-2 lines (Fig. 4*B*), in the MCF-7/MEK-EE transfectant, M2, and in the empty vector control line, C2 (Fig. 4*C*). Equal amounts of p27 protein were immunoprecipitated from the indicated cell lines and boiled to release associated proteins, and then heat-stable p27 was tested for its ability to inhibit a fixed amount of recombinant cyclin E1-cdk2. The cyclin E1-cdk2 complexes were then immunoprecipitated, and kinase activity was assayed. The activity of the p27-treated cyclin E1-cdk2 was expressed as a % of control, uninhibited cyclin E1-cdk2. p27 from the MCF-7 line had approximately four times the inhibitory potency as p27 from the LY-2 line (Fig. 4*B*). Similarly, MEK overexpression in the M2 line impaired the inhibitory function of p27 (Fig. 4*C*). The cyclin E1-cdk2 inhibitory activity of increasing amounts of p27 from the MEK^{EE}-transfected M2 line was compared with that of p27 from the vector alone control, C2. Even a 3-fold (3×) excess of p27 in the M2 line did not achieve the same level of cyclin E1-cdk2 inhibition shown by p27 (1×) from the control line.

MEK Inhibition Modulates p27 Phosphorylation—Our antisense experiments showed that p27 is essential for the antiestrogen arrest of LY-2 following partial MEK inhibition (Fig. 3*D*). Since MEK inhibition restored antiestrogen arrest, we postulated that MEK inhibition might alter p27 phosphorylation. As seen in asynchronously proliferating cells (Fig. 2*E*), the 2D-IEF of p27 from antiestrogen-treated MCF-7 and LY-2 cells showed five distinct p27 isoforms (labeled 1–5 in Fig. 5*A*) with isoforms 1 and 3 again being the most abundant. p27 from antiestrogen-arrested MCF-7 showed a predominance of isoform 1, with the ratio of isoforms 1 to 3 being 2:1. In antiestrogen-treated LY-2 cells, form 3 was the predominant form, with the isoform 1:isoform 3 ratio at 1:2. Treatment with 0.1 μM U0126 together with either 4-OH-TAM or ICI changed the p27 phosphorylation profile in LY-2 cells to one that more closely resembled that in antiestrogen-arrested MCF-7 cells, with the p27 isoform 1 more abundant than isoform 3 at a ratio of 2:1 (data shown for ICI treatment in Fig. 5*A*). In all cell types, ICI treatment increased the relative abundance of isoforms 4 and 5 compared with that of untreated cells.

We tested whether the changes in total cellular p27 phosphorylation were reflected by changes in the phosphorylation of cyclin E1-bound p27 (Fig. 5*B*). Cyclin E1-bound p27 in the ICI-treated LY-2 line showed a predominance of isoform 3 (the ratio of isoform 1:isoform 3 was 1:6 by densitometry), whereas LY-2 cells arrested by the combination of MEK inhibition and antiestrogen showed a cyclin E1-associated p27 phosphorylation pattern more closely resembling that in antiestrogen-arrested MCF-7 (isoform 1:isoform 3 ratio nearly 1:1 in both). These data suggest that the combination of MEK inhibition and antiestrogen treatment may restore the cyclin E1-cdk2 inhibitory function of p27 in LY-2, at least in part, by altering p27 phosphorylation.

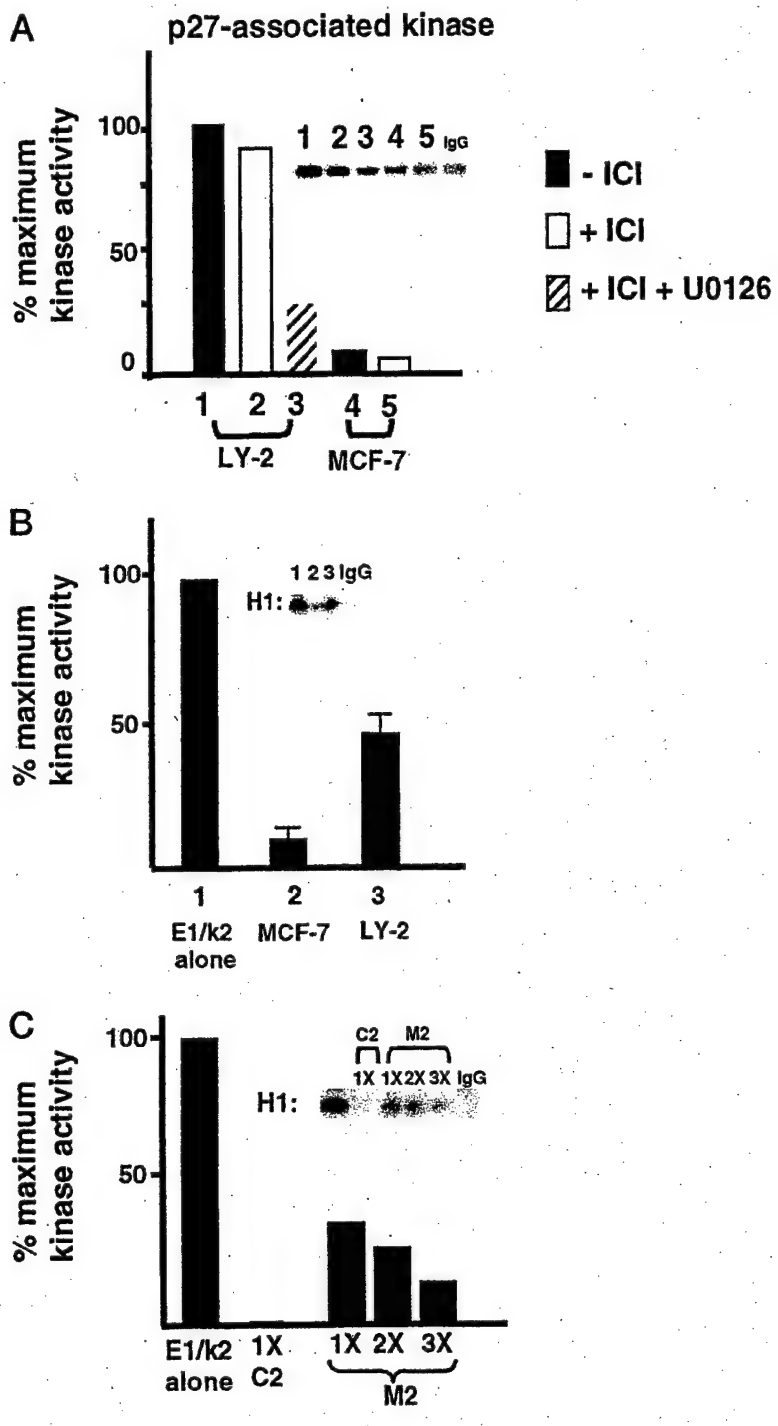

FIG. 4. **MEK inhibition partially restores p27 inhibitory function and changes the pattern of p27 phosphorylation.** *A*, equal amounts of p27 were precipitated from asynchronous (*lanes 1, 4*) and ICI-treated LY-2 and MCF-7 cells (*lanes 2, 5*) and from LY-2 cells treated with both U0126 + ICI (*lane 3*) and analyzed for associated histone H1 kinase activity. Radioactivity in the histone H1 substrate was quantitated by PhosphorImager and expressed as a percentage of maximum activity after subtraction of background from IgG control (*lane 6*) and graphed. The *inset* shows the autoradiogram of activity in histone H1. The data presented are the mean of repeat experiments. *B*, p27 was immunoprecipitated from asynchronously proliferating MCF-7 and LY-2 lysates and boiled, and equal amounts of p27 were added to recombinant cyclin E1-cdk2. Cyclin E1 complexes were then immunoprecipitated and assayed for histone H1 (*H1*) kinase activity. Inhibition of cyclin E1-cdk2 activity by added p27 is shown. Radioactivity incorporated into the histone H1 substrate is shown in the autoradiograph (see *inset, upper right*) and graphed as % maximum uninhibited cyclin E1-cdk2 activity. Mouse IgG served as an immunoprecipitation control. *C*, equal amounts of p27 were immunoprecipitated from the asynchronously proliferating MCF-7/MEK^{EE} clone (*M2*) or from the empty vector control (*C2*) and cyclin E1-cdk2 inhibitor function assayed as in *B*.

DISCUSSION

The key roles of p21 and p27 in antiestrogen arrest have been demonstrated in earlier studies (3, 48). Antiestrogens increase cyclin E1-cdk2-KIP binding, and immunodepletion of p21 and p27 from steroid-depleted or tamoxifen-arrested cells removes essentially all cellular cyclin E1-cdk2 (49–51), suggesting that these cyclin complexes are fully saturated by p21 or p27 in arrested cells. Recent work with antisense (AS) p27 and p21 demonstrated that inhibition of expression of either KIP from antiestrogen-arrested cells leads to cell cycle re-entry (3, 48). In addition to increased KIP-cdk binding, other cell cycle effectors contribute to G_1 arrest by antiestrogens. These include reductions in c-Myc and cyclin D1 and Cdc25A, increased p15, and potentially, the accumulation of cdk2 in a non-CAK-activated form (3, 50–53). However, while induction of cell cycle arrest by antiestrogens has multiple effectors, the antisense studies demonstrate that KIP function is required for maintenance of arrest. Moreover, the present work indicates that deregulation of p27 inhibits antiestrogen responsiveness.

Our data suggest that constitutive MEK/MAPK activation contributes to the development of antiestrogen resistance in ER-positive breast cancer cells, at least in part, by compromising the inhibitory function of p27. We show here that a non-cytostatic and non-cytotoxic dose of the MEK inhibitor, U0126, restored sensitivity to G_1 arrest by antiestrogens in the widely used LY-2 model of antiestrogen resistance. Moreover, transfection of *HER2* or MEK^{EE} into MCF-7 impaired antiestrogen responses. In antiestrogen-treated LY-2 and MCF-7/MEK^{EE} transfectants, p27 failed to accumulate in cyclin E1-cdk2 complexes and did not inhibit this kinase. MEK inhibition by

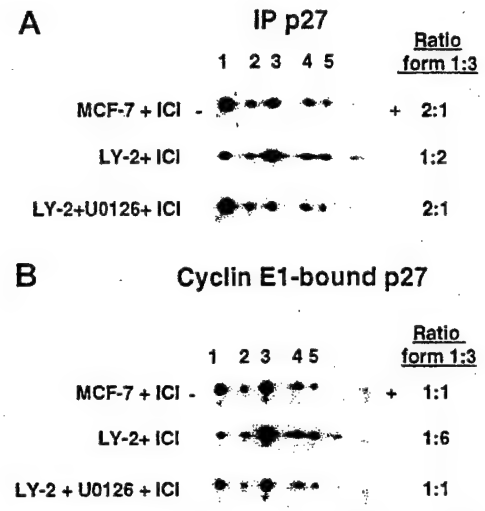

FIG. 5. **MEK inhibition modulates p27 phosphorylation.** *A* and *B*, the 2D-IEF patterns of immunoprecipitated p27 *(A)* or cyclin E1-bound p27 *(B)* were assayed in MCF-7 and LY-2 cells after 48 h of treatment with ICI, and in LY-2 cells after 48 h treatment with both ICI and UO126.

U0126 in these antiestrogen-resistant lines altered p27 phosphorylation and restored the inhibitory binding of p27 to cdk2 following antiestrogen treatment. Thus, MEK/MAPK-dependant p27 phosphorylation events are associated with a reduced ability to inhibit cdk2.

Through the course of selection of the antiestrogen-resistant LY-2, p27 regulation has been altered such that its binding to cyclin E1-cdk2 is increased without a commensurate reduction in cyclin E1-cdk2 activity. In antiestrogen-mediated arrest of MCF-7, a 3-fold increase in p27 binding to cyclin E1-cdk2 is sufficient for cdk2 inactivation and cell cycle arrest (3). The approximately 4-fold increase in cyclin E1-bound p27 in asynchronously growing LY-2 cells relative to that in proliferating MCF-7 and the failure of antiestrogens to increase p27 binding to cyclin E1-cdk2 in LY-2 prompted further investigation of p27 function in these lines. Indeed, both cyclin E1 and p27 immunoprecipitates contain detectable histone H1 kinase activity in LY-2 cells. While the p27-associated kinase activity could reflect dissociation of p27 from cyclin E1-cdk2 *in vitro* following immunoprecipitation of the complexes, the increased binding of p27 to cyclin E1 without loss of kinase activity in asynchronous LY-2 suggests that some of the cyclin E1-cdk2-p27 complexes may retain activity *in vivo*. Detection of p27-immunoprecipitable kinase activity has been reported by others (54, 55). The elevated level of p27 protein in the LY-2 line may reflect MAPK-independent events that have occurred throughout the course of selection of this line (44).

p27 levels were reduced in the MEK^{EE} transfectants, consistent with the observation by others that Ras-MAPK contributes to p27 degradation (15, 20, 56). Despite the lower total p27 protein levels in these cells, cyclin E1-bound p27 levels were not reduced. Moreover, while cyclin E1-bound p21 and p27 levels were similar in MEK^{EE} transfectants and control lines, cyclin E1-cdk2 activity was increased in asynchronously proliferating MEK^{EE} transfectants compared with controls. Thus, MAPK activation at the levels achieved here, may favor the association of p27 with cdk2 in a poorly inhibitory form, such that some of the cyclin E1-cdk2-p27 complexes retain activity. This effect may be separable from the effect of MAPK on p27 stability. p27 forms that can bind cyclin E1-cdk2 but fail to inhibit this kinase have been modeled previously *in vitro* (8). Further evidence to support functional alteration of p27 in the LY-2 and MEK^{EE} transfected MCF-7 lines is provided by assays of p27 inhibitory function. p27 from both LY-2 and the MEK^{EE}-transfected line, M2, both showed a reduced ability to inhibit recombinant cyclin E1-cdk2 *in vitro*. As an alternate model, MEK/MAPK activation may reduce the stability with which p27 binds to cyclin E1-cdk2 *in vitro*, allowing detection of this kinase activity in p27 immunoprecipitates through dissociation *in vitro*. A reduced ability to bind cyclin E1-cdk2 could also account for the reduced inhibitory activity of heat-stable p27 isolated from the resistant lines.

Signal transduction pathways have been shown to affect p27 inhibitory function (15, 57), raising the possibility that phosphorylation events may modulate p27 function. Overexpression of the integrin-linked kinase causes a reduction in inhibitory activity of p27 toward cyclin E1-cdk2 (57). Here we showed that the antiestrogen ICI182780 modulates p27 phosphorylation in MCF-7 cells, increasing the relative amounts of p27 isoforms 3, 5, and 6. We also showed an association between altered p27 inhibitory function and altered phosphorylation in LY-2 and MCF-7/MEK^{EE} cells, suggesting that deregulated p27 phosphorylation may be causally linked to antiestrogen resistance. Although MAPK can phosphorylate p27 *in vitro* (15–17), it is not known at present whether direct phosphorylation of p27 by MAPK occurs *in vivo*. The effects of MAPK on the p27 phosphorylation profile may be indirect. p27 contains several potential MAPK consensus sites, including serine 10 (Ser-10), Ser-178, and Thr-187. Ser-10 has recently been shown to be a major p27 phosphorylation site in G_0-arrested cells, although it may not be a physiological MAPK target site (16). Since a p27 mutation converting Ser-10 to alanine or aspartate did not affect the ability of p27 to inhibit cyclin E1-cdk2 *in vitro* (16), the MAPK-dependent pathway that modulates both p27 phosphorylation and its ability to inhibit cdk2 cannot uniquely affect Ser-10. Moreover, the phosphorylation of p27 at Thr-187 that regulates its recognition by the F box protein Skp-2, does not affect the cdk2 inhibitory function of p27 (8). Thus, phosphorylation at sites other than Ser-10 and Thr-187 may be required for the MEK/MAPK-dependent phosphorylation of p27 that modulates its cdk2 inhibitory function. The identity of the different 2D-IEF phospho-isoforms of p27 observed by 2D-IEF warrants further investigation.

The causes of MAPK activation in human cancers differ among different tumors. MAPK activation is increased in up to 50% of breast cancers compared with normal breast epithelium and is associated with poor patient prognosis (58–60). HER-2/ErbB-2 overexpression, seen in up to 30% of breast cancers is often associated with antiestrogen resistance (27). HER-2/ErbB-2 signaling has been shown to decrease p27 stability via MAPK activation (56). In the HER-2 overexpressing MCF-7/HER-2–18, MEK inhibition by U0126 restored sensitivity to antiestrogens. Taken together, the present study links HER-2/ErbB-2 activation and antiestrogen resistance through MAPK-dependent alterations in p27 function.

In addition to its mechanistic relevance to breast cancer, the observed link between p27 dysfunction and MAPK activation has implications for many types of cancers. The reduced levels of p27 observed in many cancers (colon, lung, prostate, gastric) may reflect oncogenic activation of the Ras/MEK/MAPK pathway (12). For example, the increased p27 proteolytic activity observed in colon cancer lysates may result from oncogenic activation of *K-Ras* in these cancers (61). There is a strong molecular rationale supporting the continued development of MEK/MAPK inhibitory drugs. A number of MEK inhibitors have shown good oral bioavailability and efficacy in preclinical trials (62). Tumor-specific MEK inhibitors may have the potential to restore p27 protein levels and inhibitory function and thereby restrain tumor growth.

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REFERENCES

1. Sherr, C. J., and Roberts, J. M. (1999) *Genes Dev.* **13,** 1501–1512
2. Coats, S., Flanagan, M., Nourse, J., and Roberts, J. M. (1996) *Science* **272,** 877–880
3. Cariou, S., Donovan, J., Flanagan, W., Milic, A., Bhattacharya, N., and Slingerland, J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 9042–9046
4. Hengst, L., and Reed, S. I. (1996) *Science* **271,** 1861–1864
5. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. (1995) *Science* **269,** 682–685
6. Millard, S. S., Yan, J. S., Nguyen, H., Pagano, M., Kiyokawa, H., and Koff, A. (1997) *J. Biol. Chem.* **272,** 7093–7098
7. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M., and Clurman, B. E. (1997) *Genes Dev.* **11,** 1464–1478
8. Vlach, J., Hennecke, S., and Amati, B. (1997) *EMBO J.* **16,** 5334–5344
9. Ponce-Castaneda, M. V., Lee, M.-H., Latres, E., Polyak, K., Lacombe, L., Montgomery, K., Mathew, S., Krauter, K., Sheinfeld, J., Massague, J., and Cordon-Cardo, C. (1995) *Cancer Res.* **55,** 1211–1214
10. Pietenpol, J. A., Bohlander, S. K., Sato, Y., Papadopoulos, N., Liu, B., Friedman, C., Trask, B. J., Roberts, J. M., Kinzler, K. W., Rowley, J. D., and Vogelstein, B. (1995) *Cancer Res.* **55,** 1206–1210
11. Tan, P., Cady, B., Wanner, M., Worland, P., Cukor, B., Magi-Galluzzi, C., Lavin, P., Draetta, G., Pagano, M., and Loda, M. (1997) *Cancer Res.* **57,** 1259–1263
12. Slingerland, J., and Pagano, M. (2000) *J. Cell. Physiol.* **183,** 10–17
13. Aktas, H., Cai, H., and Cooper, G. M. (1997) *Mol. Biol. Cell* **17,** 3850–3857
14. Cheng, M., Sexl, V., Sherr, C. J., and Roussel, M. F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1091–1096
15. Kawada, M., Yamagoe, S., Murakami, Y., Suzuki, K., Mizuno, S., and Uehara, Y. (1997) *Oncogene* **15,** 629–637
16. Naumann, U., Weit, S., Rieger, L., Meyermann, R., and Weller, M. (1999) *Biochem. Biophys. Res. Commun.* **261,** 890–896
17. Ishida, N., Kitagawa, M., Hatakeyama, S., and Nakayama, K. (2000) *J. Biol. Chem.* **275,** 25146–25154
18. Cobb, M. H. (1999) *Prog. Biophys. Mol. Biol.* **71,** 479–500
19. Leone, G., DeGregori, J., Sears, R., Jakoi, L., and Nevins, J. R. (1997) *Nature* **387,** 422–426
20. Takuwa, N., and Takuwa, Y. (1997) *Mol. Cell. Biol.* **17,** 5348–5358
21. Maemura, M., Iino, Y., Koibuchi, Y., Yokoe, T., and Morishita, Y. (1999) *Oncology* **57** Suppl. **2,** 37–44
22. Nicholson, S., Wright, C., Sainsbury, J. R., Halcrow, P., Kelly, P., Angus, B., Farndon, J. R., and Harris, A. L. (1990) *J. Steroid Biochem. Mol. Biol.* **37,** 811–814
23. Wright, C., Angus, B., Nicholson, S., Sainsbury, J. R., Cains, J., Gullick, W. J., Kelly, P., Harris, A. L., and Horne, C. H. (1989) *Cancer Res.* **49,** 2087–2090
24. Press, M. F., Pike, M. C., Chazin, V. R., Hung, G., Udove, J. A., Markowicz, M., Danyluk, J., Godolphin, W., Sliwkowski, M., and Akita, R., *et al.* (1993) *Cancer Res.* **53,** 4960–4970
25. Borg, A., Baldetorp, B., Ferno, M., Killander, D., Olsson, H., Ryden, S., and Sigurdsson, H. (1994) *Cancer Lett.* **81,** 137–144
26. Nicholson, S., Sainsbury, J. R., Halcrow, P., Chambers, P., Farndon, J. R., and Harris, A. L. (1989) *Lancet* **1,** 182–185
27. Houston, S. J., Plunkett, T. A., Barnes, D. M., Smith, P., Rubens, R. D., and Miles, D. W. (1999) *Br. J. Cancer* **79,** 1220–1226
28. Coutts, A. S., and Murphy, L. C. (1998) *Cancer Res.* **58,** 4071–4074
29. El-Ashry, D., Miller, D., Kharbanda, S., Lippman, M. E., and Kern, F. G. (1997) *Oncogene* **15,** 435
30. Kurokawa, H., Lenferink, A. E., Simpson, J.F., Pisacane, P. I., Sliwkowski, M. X., Forbes, J. T., and Arteaga, C. L. (2000) *Cancer Res.* **60,** 5887–5894
31. Fisher, B., Constantino, J., Redmond, C., Poisson, R., Bowman, D., Couture, J., Dimitrov, N. V., Wolmark, N., Wickerham, D. L., Fisher, E. R., Morgolese, R., Robidoux, A., Shibata, H., Terz, J., Paterson, A. H. G., Feldman, M. I., Farrar, W., Evans, J., Lickley, H. L., and Ketner, M. (1989) *N. Engl. J. Med.* **320,** 479–484
32. Fisher, B., Costantino, J. P., Wickerham, D. L., Redmond, C. K., Kavanah, M., Cronin, W. M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J., Daly, M., Wieand, S., Tan-Chiu, E., Ford, L., and Wolmark, N. (1998) *J. Natl. Cancer Inst.* **90,** 1371–1388
33. Novotny, L., Rauko, P., Vachalkova, A., and Peterson-Biggs, M. (2000) *Neoplasma* **47,** 3–7
34. Jordan, V. C. (1995) *Breast Cancer Res. Treat.* **36,** 267–285
35. Robertson, J. F. R. (1996) *Br. J. Cancer* **73,** 5–12
36. Fuqua, S. A., Chamness, G. C., and McGuire, W. L. (1993) *J. Cell. Biochem.* **51,** 135–139
37. Osborne, C. K., Coronado, E., Allred, D. C., Wiebe, V., and DeGregorio, M. (1991) *J Natl Cancer Inst* **83,** 1477–1482
38. Takimoto, G. S., Graham, J. D., Jackson, T. A., Tung, L., Powell, R. L., Horwitz, L. D., and Horwitz, K. B. (1999) *J Steroid Biochem. Mol Biol* **69,** 45–50
39. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon. (1995) *Science* **270,** 1491–1494
40. Catzavelos, C., Bhattacharya, N., Ung, Y. C., Wilson, J. A., Roncari, L., Sandhu, C., Shaw, P., Yeger, H., Morava-Protzner, I., Kapusta, L., Franssen, E., Pritchard, K. I., and Slingerland, J. M. (1997) *Nature Med.* **3,** 227–230
41. Porter, P. L., Malone, K. E., Heagerty, P. J., Alexander, G. M., Gatti, L. A., Firpo, E. J., Daling, J. R., and Roberts, J. M. (1997) *Nature Med.* **3,** 222–225
42. Soule, H. D., Vazquez, J., Long, A., Albert, S., and Brenan, S. (1973) *J. Natl. Cancer Inst.* **51,** 1409–1413
43. Bronzert, D. A., Greene, G., and Lippman, M. E. (1985) *Endocrinology* **117,** 1409–1417
44. Brunner, N., Frandsen, T. L., Holst-Hansen, C., Bei, M., Thompson, E. W., Wakeling, A. E., Lippman, M. E., and Clarke, R. (1993) *Cancer Res.* **53,** 3229–3232
45. Sandhu, C., Garbe, J., Daksis, J., Pan, C.-H., Bhattacharya, N., Yaswen, P., Koh, J., Slingerland, J., and Stampfer, M. R. (1997) *Mol. Cell. Biol.* **17,** 2458–2467
46. Lauper, N., Beck, A. R., Cariou, S., Richman, L., Hofmann, K., Reith, W., Slingerland, M. M., and Amati, B. (1998) *Oncogene* **17,** 2637–2643
47. Slingerland, J. M., Hengst, L., Pan, C.-H., Alexander, D., Stampfer, M. R., and Reed, S. I. (1994) *Mol. Cell. Biol.* **14,** 3683–3694
48. Carroll, J. S., Prall, O. W., Musgrove, E. A., and Sutherland, R. L. (2000) *J. Biol. Chem.* **275,** 38221–38229
49. Foster, J., and Wimalasen, J. (1996) *Mol. Endocrinol.* **0,** 488–496
50. Prall, O. W. J., Sarcevic, B., Musgrove, E. A., Watts, C. K. W., and Sutherland, R. L. (1997) *J. Biol. Chem.* **272,** 10882–10894
51. Planas-Silva, M. D., and Weinberg, R. A. (1997) *Mol. Cell. Biol.* **17,** 4059–4069
52. Prall, O. W. J., Rogan, E. M., Musgrove, E. A., Watts, C. K. W., and Sutherland, R. L. (1998) *Mol. Cell. Bio.* **18,** 4499–4508
53. Foster, J. S., Henley, D. C., Bukovsky, A., Seth, P., and Wimalasena, J. (2001) *Mol. Cell., Biol.* **21,** 794–810
54. LaBaer, J., Garrret, M., Steenson, M., Slingerland, J., Sandhu, C., Chou, H., Fattaey, A., and Harlow, H. (1997) *Genes Dev.* **11,** 847–862
55. Florenes, V. A., Bhattacharya, N., Bani, M. R., Ben-David, J., Kerbel, R. S., and Slingerland, J. M. (1996) *Oncogene* **13,** 2447–2457
56. Yang, H.-Y., Zhou, B. P., Hung, M.-C., and Lee, M.-H. (2000) *J. Biol. Chem.* **275,** 24735–24739
57. Radeva, G., Petrocelli, T., Behrend, E., Leung-Hagesteijn, C., Filmus, J., Slingerland, J., and Dedhar, S. (1997) *J. Biol. Chem.* **272,** 13937–13944
58. Hori, M., Inagawa, S., Shimazaki, J., and Itabashi, M. (2000) *Pathol. Res. Pract.* **196,** 817–826
59. Mueller, H., Flury, N., Eppenberger-Castori, S., Kueng, W., David, F., and Eppenberger, U. (2000) *Int. J. Cancer* **89,** 384–388
60. Salh, B., Marotta, A., Matthewson, C., Ahluwalia, M., Flint, J., Owen, D., and Pelech, S. (1999) *Anticancer Res.* **19,** 731–740
61. Loda, M., Cukor, B., Tam, S. W., Lavin, P., Fiorentino, M., Draetta, G. F., Jessup, J. M., and Pagano, M. (1997) *Nat. Med.* **3,** 231–234
62. Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Tecle, H., Barrett, S. D., Bridges, A., Przybranowski, S., Leopold, W. R., and Saltiel, A. R. (1999) *Nat. Med.* **5,** 810–816

CRM1/Ran-mediated nuclear export of $p27^{Kip1}$ involves a nuclear export signal and links p27 export and proteolysis

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Abstract

We show that p27 localization is cell cycle regulated and suggest that active CRM1/RanGTP-mediated nuclear export of p27 may be linked to cytoplasmic p27 proteolysis in early G1. p27 is nuclear in G0 and early G1 and appears transiently in the cytoplasm at the G1/S transition. Association of p27 with the exportin CRM1 was minimal in G0 and increased markedly during G1-to-S phase progression. Proteasome inhibition in mid G1 did not impair nuclear import of p27, but led to accumulation of p27 in the cytoplasm, suggesting that export precedes degradation for at least part of the cellular p27 pool. p27-CRM1 binding and nuclear export were inhibited by S10A mutation but not by T187A mutation. A putative NES in p27 is identified whose mutation reduced p27-CRM1 interaction, nuclear export and p27 degradation. LMB did not inhibit p27-CRM1 binding, nor did it prevent p27 export *in vitro* or in heterokaryon assays. Pre-binding of CRM1 to the HIV-1 Rev NES did not inhibit p27-CRM1 interaction, suggesting that p27 binds CRM1 at a non-LMB sensitive motif. LMB increased total cellular p27 and may do so indirectly though effects on other p27 regulatory proteins. These data suggest a model in which p27 undergoes active, CRM1-dependent nuclear export and cytoplasmic degradation in early G1. This would permit the incremental activation of cyclin E-Cdk2 leading to cyclin E-Cdk2 mediated T187 phosphorylation and p27 proteolysis in late G1 and S phase.

INTRODUCTION

The Cdk inhibitor p27 is an important regulator of G1 progression. It is highly expressed in G0, where it binds tightly and inhibits cyclin E-Cdk 2 (Slingerland *et al.*, 1994;Hengst *et al.*, 1994;Polyak *et al.*, 1994). In mid-G1, p27 also plays a role in the assembly and nuclear import of D-type cyclin-Cdk complexes (LaBaer *et al.*, 1997;Cheng *et al.*, 1999). p27 levels are regulated by translational controls and by proteolysis, and decrease as cells progress from G1 to S phase (Hengst and Reed, 1996;Millard *et al.*, 1997;Slingerland and Pagano, 2000). The ubiquitin dependent proteolysis of p27 (Pagano *et al.*, 1995) is regulated by its phosphorylation at threonine 187 (T187) by cyclin E-Cdk 2 in late G1 and S phase (Pagano *et al.*, 1995;Sheaff *et al.*, 1997;Vlach *et al.*, 1997;Montagnoli *et al.*, 1999). T187 phosphorylation allows recognition of p27 by its SCF-type E3 ligase, comprised of Skp1, Cul1, and the F-box protein, Skp2 and Roc1 and the Cks1 cofactor (Ohta *et al.*, 1999;Carrano *et al.*, 1999;Tsvetkov *et al.*, 1999;Sutterluty *et al.*, 1999;Spruck *et al.*, 2001;Ganoth *et al.*, 2001). Recent evidence suggests that p27 proteolysis is regulated by at least two distinct mechanisms, with mitogenic signaling conditioning p27 for degradation in early G1 in a manner independent of T187 phosphorylation (Malek *et al.*, 2001;Hara *et al.*, 2001), while Skp2 dependent cyclin E-Cdk 2-mediated degradation occurs in S phase following T187 phosphorylation (Malek *et al.*, 2001).

While p27 is detected in the nuclei of most normal quiescent cells (Slingerland and Pagano, 2000), the relationship between its intracellular localization and proteolysis has afforded some controversy. Efficient degradation of mammalian p27 and of its Xenopus homologue, Xic1, requires nuclear import to allow its phosphorylation by cyclin E-Cdk2 (Guan *et al.*, 2000;Swanson *et al.*, 2000). Interaction of p27 with the nuclear pore protein, Nup50/NPAP60 (Muller *et al.*, 2000;Guan *et al.*, 2000) may play a role in p27 import. A nuclear localization

signal has been identified and import may also involve the binding of p27 by other import mediators (Reynisdottir and Massague, 1997;Zeng *et al.*, 2000). Nup50 antibodies have been shown to block nuclear export but not import (Guan *et al.*, 2000), thus a potential role for p27-Nup50 interaction in p27 export cannot be excluded (Smitherman *et al.*, 2000). p27 can interact with the Jun activation domain-binding protein 1 ($p38^{Jab1}$). Cotransfection of p27 together with Jab1 led to accelerated proteolysis of p27. The observation that the cytotoxin leptomycin B (LMB) inhibited Jab1-mediated p27 proteolysis suggested that CRM1-dependent nuclear export mechanisms influence p27 degradation (Tomoda *et al.*, 1999) and provided a potential link between p27 turnover and nuclear export. In contrast, the Xenopus homologue of p27, Xic1, is ubiquitinated in the nucleus and its proteolysis is not impaired by LMB (Swanson *et al.*, 2000).

The nuclear pore has an estimated mass of ≈125 MDa and controls nucleo-cytoplasmic protein exchange. Although the pore diameter permits molecules of up to 50 kDa to diffuse freely across the nuclear envelope, the localization of many small proteins is actively regulated (Mattaj and Engimeier, 1998;Gorlich and Kutay, 1999). Proteins whose function is spatially and temporally regulated, such as cyclin D1 (Alt *et al.*, 2001), cyclin B1 (Pines and Hunter, 1991;Pines and Hunter, 1994;Yang *et al.*, 1998;Jin *et al.*, 1998), IκB (Huang *et al.*, 2000) and MAPK (Adachi *et al.*, 2000), are actively transported between the nucleus and cytoplasm. This process involves a number of nucleo-cytoplasmic shuttling proteins. Two proteins, CRM1/exportin 1 (Fukuda *et al.*, 1997;Stade *et al.*, 1997;Fornerod *et al.*, 1997) and the small *ras* family GTPase, Ran (Melchior *et al.*, 1993;Moore and Blobel, 1993), both play prominent roles in the regulation of nuclear protein export. Ran is abundant and exists in GDP- or GTP-bound forms depending on its cellular location (Melchior and Gerace, 1998;Macara, 1999;Azuma and Dasso, 2000;Sacer and Dasso, 2000). Cytoplasmic RanGDP plays a key role in nuclear import.

In the nucleus, RanGDP is converted to RanGTP by the guanine nucleotide exchange factor RCC1, and becomes competent to bind export cargo prior to shuttling back to the cytoplasm (Nigg, 1997;Mattaj and Engimeier, 1998;Gorlich and Kutay, 1999). The exportin protein, CRM1 binds export cargo proteins and RanGTP in the nucleus to form an export complex that is subsequently translocated to the cytoplasm (Stade *et al.*, 1997;Fornerod *et al.*, 1997;Kehlenbach *et al.*, 1998). At the cytoplasmic face, the complex is dissociated by RanGAP, in combination with either RanBP1 or RanBP2 (Nigg, 1997;Mattaj and Engimeier, 1998;Gorlich and Kutay, 1999).

In the present study, we demonstrate that nuclear export of the Cdk inhibitor, p27, is actively regulated by CRM1/RanGTP binding. Nuclear export of p27 is time-, temperature- and energy-dependent. Although p27 binding to CRM1 is cell cycle regulated, it is LMB insensitive and LMB has no apparent effect on CRM1-mediated p27 nuclear export either *in vitro* or *in vivo*. A putative nuclear export sequence (NES) in p27 is identified. Mutation of this NES reduced nuclear export, impaired p27-CRM1 interactions and increased p27 stability.

MATERIALS AND METHODS

Cell culture. MCF-7 cells were grown in improved modified Eagle's medium (IMEM) supplemented with 5% (vol/vol) fetal bovine serum (FBS). Cells were synchronized in G0 by estradiol depletion as described (Cariou *et al.*, 2000). Cells were released from quiescence by the addition of 10^{-8} M 17β-estradiol (Sigma).

Plasmids and transfections. A vector encoding wild type p27 fused to an enhanced yellow-green variant of the Aequorea victoria green fluorescent protein (YFPp27 WT) was prepared by insertion of the full-length wild type human p27 cDNA sequence into the pEYFP-C1 vector (Clontech). Double L41A/L45A mutations were introduced into the YFPp27 WT vectors using a Quickchange site-directed mutagenesis kit (Stratagene) to generate the YFPp27NES vector. All p27 cDNA constructs were sequenced fully to ensure the absence of cloning artifacts. Asynchronous MCF-7 cells were transfected with the different YFPp27 vectors (10 μg) using lipofectamine PLUS™ (GIBCO) and then estradiol-deprived for 48 hours. Assays of YFPp27 nuclear export and binding to CRM1 were carried out at 48 hours post-transfection. Equal transfection efficiency was verified by direct visualization of p27 by fuorescence microscopy. For the heterokaryon assays, we used a green fluorescence protein tagged p53 construct kindly provided by G. Wahl (Stommel *et al.*, 1999).

Indirect immunofluorescence of p27 and BrdU. MCF-7 cells were grown on glass slides, arrested in G0 by estradiol depletion (48 hours) and then induced to re-enter the cell cycle by the addition of 10^{-8} M 17β-estradiol. Cells were also labeled with 5-bromo-2-deoxy uridine (BrdU) to monitor the timing of S phase entrance. In G0 and at intervals after estradiol addition, cells were fixed in 4% paraformaldehyde and 0.2% Triton X-100, blocked in 3% H_2O_2 and 10% goat serum and then incubated with a mouse monoclonal antibody for p27 (Transduction

Laboratories) and a rabbit polyclonal anti-BrdU antibody (CalTag Laboratories). Fluorescein (FITC) conjugated anti-mouse and texas red conjugated anti-rabbit secondary antibodies allowed visualization of p27 protein and BrdU incorporation, respectively. Cells were visualized by confocal fluorescence microscopy and photographed.

Detection of p27-CRM1 complexes in cell lysates. At indicated times after G0 release, p27 was immunoprecipitated from 1mg cellular lysate using the C-19 antibody (Santa Cruz Biotechnology). Complexes were resolved and associated CRM1 detected by immunoblotting with polyclonal CRM1 antibodies (kindly provided by R. Kehlenbach and L. Gerace or by M Fornerod) (Fornerod *et al.*, 1997;Kehlenbach *et al.*, 1998). p27 was detected in CRM1 immunoprecipitaes from 1 mg cell lysate using the Gerace lab anti-CRM1 antibody for immunoprecipitation.

Detection of p27-Cyclin E1-Cdk2 complexes. To test the effect of NES mutation on the association of p27 with cyclin E1-cdk2, cells were transfected with either YFPp27WT or YFPp27NES and cyclin E1 or YFP proteins immunoprecipitated. Complexes were resolved by SDS-PAGE and immunoblotted to detect associated proteins (cyclin E1 or p27).

Subcellular fractionation and *in vitro* nuclear export. Cells in G0 and early G1 were harvested and resuspended in 300 µl of an isotonic transport buffer (20mM HEPES pH 7.3, 110mM KAcetate, 5mM NaAcetate, 2mM MgAcetate, 1mM EGTA and 2mM DTT) containing protease inhibitors, 1 mM $NaVO_3$ and 50 mM NaF. Digitonin (20-30 µg/ml final concentration) was added until 90-95% of the cells exhibited trypan blue staining. Cells were then centrifuged and the supernatant (cytoplasmic fraction) recovered. The pellet (nuclear fraction) was washed and resuspended in 300 µl of transport buffer. The intact nuclei were then used for nuclear export assays, or for immunoblotting. To assay nuclear export of p27, digitonin-permeabilized

cells were incubated with fractionated cytosolic protein (75 μg), an ATP regenerating system (5 mM ATP, 5 mM creatine phosphate, 20 U/ml creatine phosphokinase) and 2 mM GTP at room temperature for up to 30 minutes (Adam *et al.*, 1992). Nuclear export was stopped by centrifugation and the supernatant was removed. p27 was immunoprecipitated from both nuclear and supernatant fractions, resolved by SDS-PAGE and nuclear and exported p27 was detected by immunoblotting. For controls, export assays were conducted at 4°C and in the absence of cytosolic proteins, an ATP regenerating system or both.

For detection of nuclear p27 export using indirect immunofluorescence, cells were grown on glass slides and permeabilized by incubation with 25 μg/ml digitonin for 3 min at 4°C. The cells were washed and incubated with cytosolic proteins (2.5 mg/ml) and an ATP regenerating system (as above) at room temperature for 10, 20 and 30 min. Nuclear p27 was detected by indirect immunofluorescence and fluorescence intensity quantified using Carl Zeis Laser Scanning Software (LSM) 510 and confocal microscopy.

To assay the expression and nuclear export of green-yellow fluorescent-tagged p27, MCF-7 cells were grown on glass slides and transfected with WT or NES YFPp27 vectors. Export of p27 from digitonin permeabilized cells was as above. Nuclear export of p27 was determined by measuring the decline in nuclear YFP fluorescence by direct photomicroscopy using a digital camera and fluorescence intensity was quantified using Carl Zeis Laser Scanning Software (LSM) 510.

Heterokaryon assay. Nucleo-cytoplasmic shuttling of YFP-p27 and GFP-p53 was detected in HeLa/NIH 3T3 cell heterokaryons. Hela cells were grown on glass cover slips in DMEM containing 10% FCS and transfected using Superfect reagent (Qiagen) according to the manufacturer's instructions. Cells were washed four times with DMEM medium 12 hours after

transfection and NIH 3T3 cells were seeded. Cells were treated with cycloheximide (75 μg/ml) for 30 minutes prior to fusion. Cell fusion was induced by the addition of 50% (w/v) polyethylenglycol in DMEM for 2 min in the presence of 50 μg/ml cycloheximide. After washing 4x with PBS, cells were further incubated in DMEM containing of 50 μg/ml cycloheximide for 2 hours to allow shuttling. To the indicated samples, leptomycin B (final concentration 50 ng/ml) was added 2 hours prior to cell fusion.

After washing with PBS, cells were fixed with 3.7% formaldehyde in PBS (5 min at 20°C, 10 min 4°C). Cells were permeabilized by treatment with 0.1% Triton X in PBS (4°C) for 5 min. After washing in PBS, cells were incubated with PBS supplemented with 2% BSA at room temperature for 10 min. Actin was stained with 100 μl of Texas–Red Phalloidin (Sigma) diluted 1:2000 in PBS/2% BSA. After washing with PBS, DNA was stained with 1% Hoechst 33258 (Molecular Probes) diluted 1:2000 in PBS. Cover slips were then washed and mounted on glass slides.

p27 nuclear import. Quiescent MCF-7 cells were digitonin permeabilized and incubated with 4 μg/μl (final concentration) cytosolic proteins and an ATP-regenerating system at room temperature. His-tagged p27 was added, and the reaction incubated for 60 min. Reactions were stopped by centrifugation, and separated into nuclear and supernatant fractions. Where indicated, nuclei were pre-incubated with leptomycin B (LMB; 50 ng/ml), N-acetyl-leu-leu-norleucinal (LLnL; 2.5 μM), or wheat germ agglutinin(WGA; 20 μg/ml).

Recombinant protein assays. p27 was immunoprecipitated from 1 mg of cell lysate recovered 6 hours after estradiol stimulation of quiescent MCF-7 cells. Following 5 min incubation at 95°C to denature any endogenous p27-associated proteins, the supernatant containing heat stable p27 was incubated with recombinant CRM1, in the presence or absence of GTP-loaded Ran or

GDP-loaded Ran for 20 min at 4°C (Askjaer *et al.*, 1999). p27 was immunoprecipitated and the associated proteins separated by SDS-PAGE. To assess if p27-CRM1/Ran complexes could be dissociated *in vitro* they were incubated with recombinant RanGAP and RanBP1 for 30 min at 30°C. p27 immune complexes were then centrifuged and dissociation of CRM1 or Ran into the supernatant was assayed by immunoblotting both fractions. Similar experiments were conducted using cells transfected with the WT and mutant YFPp27 constructs.

Similar experiments were carried out entirely with recombinant proteins. Recombinant his-tagged p27 prepared in E. coli or bacculovirus produced cyclin D1 proteins were mixed with recombinant CRM1 for 60 min at 4°C with or without pre-treatment of CRM1 with LMB 100 ng/ml for 30 min. p27 or cyclin D1 was immunoprecipitated, complexes resolved and associated CRM1 detected by immunoblotting.

To test the effect of HIV-1 Rev on p27-CRM1 and cyclin D1-CRM1 complexes, the HIV-1 Rev NES peptide, NH2-CLPPELERLTL-COOH (Kudo et al., 1998) was synthesized and purified over reverse phase HPLC on a C8 column and verified by Mass spectrophotometry. A molar excess of peptide was pre-incubated with recombinant CRM1 for 30 min at 4°C prior to the addition of RanGTP and p27 or cyclin D1 proteins for a further 60 min. p27 or cyclin D1 immune complexes were then assayed for associated CRM1 by IP blots.

Microinjection of nuclei and assays of nuclear export of p27-NES-peptide coupled to BSA. Export ligand preparation followed published procedures (Melchior, 1998). In brief, fatty acid free BSA (Boehringer Mannheim) was conjugated with FITC isomer I (Molecular Probes) and purified via gel filtration. Peptides containing the putative p27 nuclear export sequence (CRNLFGPVDHEELTRDLE) were coupled to FITC-BSA via their N-terminal cysteine using the crosslinker, Sulfo-SMCC (Pierce). Microinjection of FITC-BSA-NES and FITC-BSA (0.1

mg/ml) into nuclei of adherent HeLa cells was performed with an Eppendorf Femtojet. Cells were kept at 37°C, and photomicrographs were taken at different times after injection using an inverted Olympus IX70 fluorescence microscope and a back illuminated CCD camera (Princeton Instruments).

RESULTS

p27 localization is cell cycle regulated. p27 localization was assayed at intervals across the cell cycle in synchronized MCF-7 cells (Cariou *et al.*, 2000). p27 levels and BrdU uptake were monitored by indirect immunofluorescence labeling and confocal microscopy. Quiescent MCF-7 cells exhibited strong nuclear p27 expression (G0, Fig. 1A). Nuclear p27 intensity fell progressively as cells moved through G1. At 9 hours after estradiol addition, late G1 cells showed predominantly nuclear p27, with some cells showing both cytoplasmic and nuclear p27 localization. Twelve hours after G0 release, at a time when p27 is known to undergo rapid proteolysis (Malek *et al.*, 2001), early S phase cells exhibiting both nuclear and cytosolic p27 were consistently detected, suggesting either delayed import or that nuclear p27 export exceeds import (Fig. 1A, early S). Since new p27 synthesis is dramatically decreased within hours of G0 exit (Hengst and Reed, 1996;Millard *et al.*, 1997)(Cariou and Slingerland, unpublished MCF-7 results), the latter may be more likely. During S phase (t=16 to 22 hours hrs), p27 became progressively undetectable in cells staining positive for BrdU uptake. In late S/early G2 cell populations, some cells were negative for both p27 staining and BrdU uptake (Fig. 1A, see dual negative cell indicated by white arrow). Flow cytometry of cells at each time point in Fig 1A is shown in Fig 1B.

p27-bound CRM1 increases during G1 progression. A major mechanism of nuclear export involves binding of the export cargo protein to the exportin CRM1. Cells were synchronized in parallel with those assayed in Fig. 1A above. Immunoprecipitation of cellular p27 at intervals between G0 and S phase revealed an association between p27 and CRM1 that increased during G1 as p27 levels decreased (Fig. 1B). p27 was also found to interact with Ran in early G1 in co-immunoprecipitation experiments (not shown). CRM1 levels were constant across the cell cycle.

Given the reduction in p27 in the 6 to 12 hours time points, the increase in p27-bound CRM1 is dramatic as cells move through G1 toward late G1/S.The increase in levels of p27-bound CRM1 is temporally associated with the activation of p27 proteolysis demonstrated earlier as cells move from G0 to G1 and S phase (Pagano *et al.*, 1995;Hengst and Reed, 1996;Malek *et al.*, 2001). The onset of this transient binding of p27 to CRM1 occurs prior to the increase in Skp2 protein levels observed 12 hours after the addition of estradiol (Fig. 1B). In addition, the onset of p27-CRM1 binding occurs prior to activation of cyclin E-Cdk2 in this cell line (Cariou *et al.*, 2000).

Nuclear export of p27 is actively regulated. The cell cycle dependent changes in localization of endogenous p27 led us to investigate how nuclear export of p27 is regulated. To assay nuclear export of p27, MCF-7 cells were grown on glass slides, synchronized in quiescence and released into the cell cycle. In mid-G1 (6 hours after release from quiescence) cells were treated with digitonin at concentrations that selectively permeabilize the cytoplasmic membrane and leave the nuclear membrane intact. Nuclear export of cellular p27 was detected by indirect immunofluorescence at intervals after the addition of ATP, an ATP-regenerating system and cytosolic proteins (Fig. 2A). A second method was used to demonstrate and quantitate p27 export. Nuclei were separated from cytosolic proteins by digitonin-permeabilization of cells in mid-G1. *In vitro* export of nuclear p27 was assayed by immunoblotting of nuclear p27 and p27 exported into the supernatant over time (Fig. 2B). Passive diffusion of p27 from the nuclei was not observed in the absence of ATP or at 4°C in either of these assays, indicating that p27 export is actively regulated (Fig. 2A and B). *In vitro* export of p27 from G0 nuclei proceeded at a rate 50% slower than that from mid G1 nuclei (Fig. 2B). Thus, as cells progress from G0 into mid-G1, p27 or other cofactors may be modified to facilitate nuclear export. The rate of p27 export assayed by densitometric analysis of p27 blots in Fig. 2B was similar to that measured by the

decay in nuclear p27 fluorescence quantitated by confocal microscopy using Carl Zeiss Laser Scanning software in Fig. 2A.

p27 interacts with CRM1 *in vitro*. When CRM1 binds to export cargo in association with nuclear RanGTP, cytoplasmic dissociation of the complex is stimulated by GTP hydrolysis. To test the specificity of p27-CRM1 interactions, formation of CRM1-Ran-p27 complexes was assayed *in vitro*. p27 was immunoprecipitated from mid-G1 cells, boiled to dissociate heat labile proteins and then incubated with recombinant CRM1 and either GTP-loaded Ran (Fig. 3A, lane 1) or GDP-loaded Ran (lane 2). p27-CRM1-Ran complexes were readily detected in the presence of RanGTP, but were significantly reduced in the presence of RanGDP. Non-specific binding of CRM1 or Ran to p27 antibody-bound protein A beads was not evident (lane 3). p27 binding to CRM1 alone was also detected and p27-CRM1-RanGTP complexes dissociated following incubation with RanBP1 and RanGAP (data not shown). These data support the specific association of p27, CRM1 and Ran complex formation in early G1 regulating nuclear export of p27.

LMB increases p27 levels but does not disrupt p27-CRM1 binding. Treatment of cells with LMB increased p27 levels (Fig. 3B, right panel and Fig 4C). Densitometric analysis of repeat experiments showed that approximately 18% of total cellular p27 was detected in CRM1 complexes at 9 hours after release from G0. However, LMB treatment did not appear to impair endogenous cellular p27-CRM1 interaction (Fig. 3B). To further explore this unexpected result, we assayed p27-CRM1 binding *in vitro* by two different methods. Pre-treatment of recombinant CRM1 with a molar excess of LMB *in vitro* did not impair the binding of CRM1 to heat stable p27 isolated from cells in mid-G1 (Fig. 3C), nor did it impair CRM1 binding to his-tagged recombinant p27 protein (Fig. 3D, lane 1 versus lane 2). In contrast, CRM1 binding to cyclin

D1 was impaired by LMB. CRM1 binding to cyclin D1 was assayed using flag-tagged recombinant cyclin D1, phosphorylated at T286 *in vitro* with GSK-3β to promote binding to CRM1 (Alt *et al.*, 2001). Cyclin D1-bound CRM1 was substantially reduced by pre-treatment of the recombinant CRM1 with LMB (Fig. 3D, lane 5 versus lane 6). LMB was unable to dissociate CRM1-bound p27 or cyclin D1 when it was added to these respective complexes subsequent to mixing of the recombinant proteins (lanes 3 and 7).

HIV-1 Rev NES peptide competes with CRM1 binding to cyclin D1 but not to p27. Active nuclear export involves binding of an exportin to a nuclear export signaling motif (NES) on the export substrate (Mattaj and Engimeier, 1998;Gorlich and Kutay, 1999). The exportin, CRM1 binds to classical NES-containing proteins via an LMB sensitive domain. The failure of LMB to inhibit CRM1 binding to p27 both *in vivo* and *in vitro* raised the possibility that p27 interacts with CRM1 in a novel manner. To test this, we assayed the effect of pre-binding CRM1 to the classical NES motif of the HIV-1 Rev protein on the association between p27 and CRM1 and between cyclin D1 and CRM1 *in vitro*. CRM1 was pre-incubated with a 10 amino acid peptide corresponding to the HIV Rev NES (NH2-CLPPLRLTL-COOH) for 30 minutes prior to the addition of RanGTP and either p27 or cyclin D1. Pre-incubation of CRM1 with the HIV-1 Rev NES peptide did not reduce the amount of CRM1 that bound to p27 (Fig 3E, lane1 versus lane 2). In contrast, the NES peptide reduced cyclin D1/CRM1 interaction (Fig 3E, lane 4 versus lane 5).

Proteasome inhibition and LMB increase detectable cytoplasmic p27. Recent reports suggest that p27 degradation is complex and may involve both T187-dependent and independent proteolytic mechanisms (Sheaff *et al.*, 1997;Malek *et al.*, 2001;Ishida *et al.*, 2002). If even a portion of cellular p27 undergoes nuclear export prior to cytosolic degradation, proteasome

inhibition should lead to accumulation of p27 within the cytoplasm. As shown previously (Pagano *et al.*, 1995), proteasome inhibitors increased p27 protein levels (Fig. 4A). Nuclear-cytoplasmic fractionation confirmed the data in Fig. 1A that in G0, p27 is almost exclusively nuclear (Fig. 4B). A modest reduction of p27 was notable by 9 hours after G0 release, with a minor amount of p27 in the cytoplasm. LLnL treatment in mid-G1 led to increases in both nuclear and detectable cytoplasmic p27 levels. The nuclear:cytoplasmic ratios of p27 were 9.2:1 and 4.5:1 for cells in mid G1 in the absence or presence of LLnL, respectively. Since LLnL appears not to delay either export or import of p27 (see below, Fig 5), these data are consistent with degradation of at least part of the cellular p27 pool occurring in the cytoplasm.

To further investigate the effect of LMB on p27 levels and localization, MCF-7 cells were released from quiescence for either 12 hours or for 6 hours followed by addition of 200 ng/ml LMB for a further 6 hrs and nuclear and cytoplasmic fractions were isolated. LMB treatment in mid-G1 inhibited the reduction in p27 levels that occurred in when cells progress into early S phase (Fig 4C and D). Although LMB caused an accumulation of p27, it did not sequester p27 exclusively within the nucleus (Fig. 4D). p27 levels were increased in both the nucleus and in the cytoplasm, exhibiting a pattern similar to that observed after inhibition of proteolysis by LLnL (Fig. 4B). Immunoblotting for RCC1 showed no escape of nuclear protein into the cytoplasmic fractions (not shown).

LLnL and LMB do not prevent nuclear export of p27 *in vitro or in vivo*. In the context of Jab-1 overexpression, LMB has been shown to inhibit the cytoplasmic accumulation of p27 and its proteolysis (Tomoda *et al.*, 1999). Theoretically, the detection of cytoplasmic p27 in both LLnL and LMB treated cells (Fig 4B and D) could reflect accelerated p27 export, impaired p27 import or impaired proteolysis of exported protein. To test this, the effects of both LMB and

LLnL on nuclear p27 export were assayed (Figs. 5A, B and C). MCF-7 cells were grown on glass slides and transfected with a vector encoding wild type p27 linked to a yellow fluorescence protein (YFPp27WT). Export of YFPp27WT from the nuclei of digitonin-permeabilized cells was assayed as in Fig 2A. A progressive reduction in nuclear YFPp27WT over 30 min was quantitated by fluorescence confocal microscopy using scanning laser microscopy software (Fig. 5B). The addition of LMB did not delay YFPp27WT nuclear export. Similar results were evident when export was assayed in the presence of LLnL (Fig. 5A and B). Nuclear export assays of endogenous p27 using the same method as in Fig 2B confirmed results above, with no inhibition of p27 nuclear export evident in the presence of either LMB or LLnL (not shown). Thus, neither LMB nor LLnL had a measurable effect on the rate or extent of p27 nuclear export.

Since LMB did not prevent p27-CRM1 interaction *in vivo* or *in vitro*, and LMB did not inhibit nuclear export of p27 *in vitro*, we next assayed the effect of LMB on nuclear to cytoplasmic shuttling of p27 *in vivo* in heterokaryons. Heterokaryon assays were conducted in which Hela cells, transfected with either YFPp27WT or yellow-green fluorescence protein linked p53 (GFPp53) vectors, were fused to untransfected NIH 3T3 cells (Fig. 5C). In the heterokaryons, the consistent appearance of YFPp27WT in the NIH 3T3 nucleus (white arrow) indicated that the YFPp27 was being exported from the Hela cell nucleus. Pre-treatment with 50 ng/ml LMB for 2 hours and cycloheximide for a 30 minutes prior to heterokaryon fusion had no effect on p27 shuttling (Fig. 5C, p27 –LMB and +LMB). All heterokaryons showed p27 shuttling in the absence of LMB and 95% of the heterokaryons pre-treated with LMB displayed p27 shuttling. In contrast, as shown by Stommel et al. (Stommel *et al.*, 1999), the export of GFPp53 from the Hela cell nucleus was inhibited by LMB, with 90% of the heterokaryons showing shuttling without LMB and only 25% showing shuttling in the presence of LMB (Fig.

5C, p53 –LMB versus +LMB).

Having shown that neither LLnL nor LMB appears to affect p27 export, the cytoplasmic accumulation of p27 following treatment with either drug could reflect impaired p27 nuclear import. Thus, we assayed the effect of these drugs on nuclear import of p27. Following a 60 minute incubation with 4 μg/μl cytosolic proteins and an ATP regenerating system, recombinant His-p27 was imported into the nuclei of digitonin permeabilized cells (Fig. 5D, 60 min). No import occurred at 4°C, in the absence of ATP and cytosol (not shown), or in the presence of wheat germ agglutinin (Fig 5D). Neither LMB nor LLnL impaired His-p27 nuclear import. Thus, LMB and LLnL do not impair either p27 import or export as measured by these assays and the appearance of cytoplasmic p27 following treatment with these drugs is consistent with delayed degradation of exported protein.

p27 contains an atypical nuclear export sequence. We identified a putative NES within the Cdk-binding domain of p27 between amino acids 32-45 (Fig. 6A) based on the homology of leucine spacing to a cryptic NES identified in the equine infectious anemia virus (EIAV) Rev protein (Mancuso *et al.*, 1998). The spacing of the three leucines in this region of p27 is highly conserved between species.

To obtain additional evidence that amino acids 32-45 in p27 comprise a functional NES, we tested its ability to mediate nuclear export of an unrelated protein. For this, peptides containing the putative p27 NES (CRNLFGPVDHEELTRDLE) were coupled to FITC-labeled BSA, and microinjected into nuclei of adherant HeLa cells. As is shown in Fig. 6B, a significant fraction of p27NES-FITC-BSA translocated into the cytoplasm within 45 minutes. In contrast, the nuclear localization of FITC-BSA remained unchanged. While p27-NES mediated export of p27NES-FITC-BSA is not very efficient, possibly due to competing events such as the observed

accumulation in nuclear speckles, or due to rate limiting binding partners, these findings support the interpretation that amino acids 32-45 in p27 function as an NES.

Two leucines within this region were mutated (L41A and L45A) and effects on p27 export examined. MCF-7 cells were transiently transfected with vectors encoding YFPp27WT or the putative NES mutant p27 linked to YFP (YFPp27NES) and nuclear export of p27 from digitonin-permeabilized cells was quantitated over time. Nuclear export of YFPp27NES was slower than that of YFPp27WT (Fig. 6B and C). The export kinetics of YFPp27WT were similar to those of the endogenous p27 in Fig 2.

Mutation of S10 but not of T187 affects nuclear export of p27 p27 contains several sites whose phosphorylation could influence its nuclear export. Ishida et al. recently identified serine 10 (S10) as a major phosphorylation site in G0 arrested cells (Ishida *et al.*, 2000). Mutation of S10 to alanine strongly inhibited p27 export (Fig. 6 C and D), suggesting that phosphorylation at this site is essential for nuclear export of p27. The nuclear export of YFPp27S10D was similar to that observed for YFPp27WT (not shown). Since p27 degradation in late G1 involves its phosphorylation at T187 by cyclin E-cdk 2, nuclear export of YFPp27T187A was assayed. The mutation of T187 to alanine had no effect on p27 export suggesting that phosphorylation at this site is not necessary for p27 nuclear export (Fig 6).

p27-CRM1 interaction is reduced by NES and S10A but not by T187A mutation. *In vitro* binding assays were conducted to test whether these p27 mutations affect p27-CRM1 interaction. To demonstrate the linearity of the p27-CRM1 binding reaction, increasing amounts of immunoprecipitated YFPp27WT (125-500 μg) were incubated with recombinant CRM1 and RanGTP, both in molar excess of p27 (Fig. 7A). p27-associated CRM1 increased in proportion to the amount of input p27 in the reactions. The NES mutant p27 (L41A/L45A) bound 44 % less

CRM1 *in vitro* than did YFPp27WT (Fig. 7B-C). Thus, the reduction in p27 export conferred by the NES mutation (Fig. 6) is associated with impaired binding to CRM1. Mutation of S10 to alanine also reduced p27-CRM1 binding to 40% that of p27WT. The T187A mutation of p27 did not affect either its rate of nuclear export or its binding to CRM1 (Fig. 7B-C).

Reduced export of the p27NES mutant is not due to decreased cyclin E-Cdk 2 binding. Since the p27 NES resides within its cyclin-binding domain, the increased stability of p27NES could theoretically reflect reduced binding to cyclin E-Cdk2 and thus reduced cyclin E-Cdk2-Skp2-dependent p27 proteolysis. To evaluate this, MCF-7 cells were transfected with YFPp27WT and YFPp27NES, and lysates were immunoprecipitated with either YFP or cyclin E antibodies and associated proteins detected by immunoblotting (Fig. 7D). The amount of p27NES bound to cyclin E-Cdk2 was similar to cyclin E-bound p27WT.

p27NES shows delayed cytoplasmic accumulation following proteasome inhibition. Cytoplasmic p27 is detected when proteasomal degradation is impaired (Fig. 4B). We reasoned that if the NES mutations delay p27 export *in vivo*, and if some p27 undergoes cytoplasmic degradation, p27NES should show a reduced or delayed appearance in the cytoplasm after proteasome inhibition. YFPp27NES and YFPp27WT were transfected into MCF-7 cells. Newly synthesized p27 was detected exclusively in the nuclei in over 90-95% of all cell populations between 16 to 24 hours post transfection (Fig 8A, -MG132). The proteasome inhibitor, MG132 (25 μM), was added to cells at 16 hours post transfection. Direct fluorescence microscopy at intervals after MG132 addition, showed a progressive cytoplasmic accumulation of p27WT, with 44% of cells showing cytoplasmic p27 by 8 hours after MG132 addition (Fig 8A, + MG132). YFPp27NES showed a significant delay in redistribution of from nucleus to cytoplasm compared to that of p27WT, with only 18% of the p27NES transfected cells displaying cytoplasmic p27 at

the same 8 hour time point. S10A mutation severely impaired p27 export, with only 7% of transfected cells exhibiting cytoplasmic p27 after 8 hours of MG132. In contrast, the S10D mutation did not affect the accumulation of cytoplasmic p27 (Fig 8A).

Delayed nuclear export is associated with increased p27 protein stability. The p27 protein half-lives ($t_{1/2}$) of p27WT and p27NES were compared by cycloheximide chase (Fig. 8B). At 48 hours post-transfection, both the YFPp27WT and YFPp27NES induced G0/G1 arrest as assayed by dual BrdU/PI staining and flow cytometry (not shown). Wild-type p27 exhibited a $t_{1/2}$ of 5.7 hours. The p27NES mutant was over twice as stable as p27WT, with a $t_{1/2}$ of 11.7 hours.

DISCUSSION

The reduction of p27 levels is critical for cyclin E-Cdk2 activation and G1-to-S phase progression. As cells exit G0, p27 synthesis is rapidly reduced (Hengst and Reed, 1996;Millard *et al.*, 1997) and its proteolysis is increased (Pagano *et al.*, 1995). The half-life of p27 is maximal in G0 and reduced in asynchronous cells (Pagano *et al.*, 1995;Hengst and Reed, 1996). Indeed in G1 and S phase cells, the p27 half-life is reduced five-fold compared to that in quiescence (Malek *et al.*, 2001). While much is known regarding mechanisms of ubiquitin-dependent SCF^{SKP2} mediated p27 proteolysis, the relationship between p27 localization and its degradation has been unclear.

Although molecules of up to 50 kDa can diffuse freely through nuclear pores, p27 is largely bound to multiprotein complexes. The present data suggests that both p27 import into and export from the nucleus are actively regulated. Moreover, we provide evidence that for at least part of the nuclear p27 pool, CRM1-dependent nuclear export may precede degradation. Detectable p27 is exclusively nuclear in G0 and early G1, with transient appearance in both the nucleus and cytoplasm as cells progress through G1, prior to its disappearance in late S phase. The dramatic increase in CRM1-p27 binding during G1 progression and the transient appearance of cytosolic p27 at the G1/S transition, suggested a link between nuclear export of p27 and its degradation. Furthermore, proteasome inhibition in G1 led to the appearance of cytoplasmic p27, at a time when new p27 synthesis is minimal. The timing of cellular p27-CRM1 interaction and the observation that p27 is exported more rapidly from G1 nuclei than from G0 nuclei suggest that p27, the CRM1-ran export machinery or both may undergo periodic post-translational changes to facilitate p27 export in early G1. p27 phosphorylation appears to play a critical role in this process. Not only does p27 phosphorylation differ between the nucleus and cytoplasm

(MC and JMS, unpublished), we and others have shown that the phosphorylation status of serine 10 (S10) critically regulates p27 export (Rodier *et al.*, 2001;Ishida *et al.*, 2002).

CRM1 mediates nuclear export by binding to a leucine-rich NES motif in the export substrate. The first NES identified was that of the HIV-1 Rev protein and a classical NES consensus sequence has been identified based on a conserved clustering of leucine residues (Bogerd *et al.*, 1996). We propose that amino acids 32-45 constitute a NES for p27. The spacing of the leucine residues in this putative p27 NES is identical to that in the NES of the EIAV Rev protein. This EIAV Rev sequence is functionally homologous to the NES of the HIV-1 Rev (Mancuso *et al.*, 1998). Although the leucines in the EIAV and p27 NES are less tightly clustered than those in the HIV-1 Rev NES, their spacing is completely conserved in the p27 sequence of all known species. When a peptide comprised of amino-acids 32-45 of p27 was linked to FITC-tagged BSA, the peptide directed export of this construct to the cytoplasm. The ability of this p27 peptide to direct the nuclear export of a heterologous protein is consistent with this sequence functioning as an NES. In control experiments, FITC-BSA without the p27 NES remained nuclear.

Mutations converting 2 of these 3 NES leucines to alanine reduced CRM1 binding, impaired export *in vitro* and prolonged the half-life of the mutant p27 protein. Moreover, the p27NES mutant showed delayed and reduced accumulation in the cytoplasm following proteasome inhibition. However, mutation of these leucine residues did not affect p27 binding to cyclin E-Cdk 2 and thus the stability of p27NES cannot be attributed to impaired cyclin E-Cdk2 mediated p27 degradation.

Nuclear export of the p27NES protein was impaired but not abolished. This may reflect the incomplete inhibition of CRM1-NES binding by these two leucine mutations, the

involvement of other motifs on p27 in CRM1 binding or the existence of another non-CRM1-dependent export mechanism for p27.

Surprisingly, LMB did not prevent the interaction between p27 and CRM1 *in vitro* or *in vivo* in LMB-treated cells. In addition, LMB did not inhibit p27 nuclear export from digitonin-permeabilized cells nor did it impair p27 export *in vivo* in heterokaryons. In contrast, the binding of CRM1 to cyclin D1 (Alt *et al.*, 2001) and nuclear export of another CRM1 cargo, p53 (Stommel *et al.*, 1999) were notably impaired by LMB drug concentrations that did not affect p27. LMB modifies the exportin CRM1 at C529 (Kudo *et al.*, 1999) and is thought to inhibit protein export by impairing CRM1-substrate NES interaction. The lack of effect of LMB on cellular p27-CRM1 binding and on p27 nuclear export are consistent with p27 binding to CRM1 at sites other than, or in addition to the LMB-sensitive motif at C529. Our observation that classical HIV1 Rev NES peptide, which reduced cyclin D1/CRM1 interactions, did not impair p27 binding to CRM1 *in vitro* suggests that p27 binds to a site distinct from the classical NES-binding motif on CRM1. Transport factors have been shown to bind different cargo using slightly different binding sites (Conti and Kuriyan, 2000).

The effect of LMB on p27 export may be modulated by the binding of other proteins to the p27-CRM1 complex. The ability of LMB to interfere with CRM1 binding to Rev can be modulated by the binding of other proteins (Askjaer *et al.*, 1998). Recently, Tomoda et al. demonstrated that p27 nuclear export was LMB sensitive in the presence of overexpressed $p38^{Jab1}$(Tomoda *et al.*, 2002). When the $p38^{Jab1}$ NES was mutated, effectively removing the protein from the p27-CRM1 complex, p27 export became LMB insensitive. Thus, the formation of an export-competent p27-CRM1 complex *in vivo* may involve other proteins that modulate the sensitivity of p27 nuclear export to LMB. This process may show important cell type and species

specific differences (Swanson *et al.*, 2000;Rodier *et al.*, 2001). More intensive investigation of the specific site(s) of CRM1-p27 interaction and of the composition of p27-CRM1 complexes is warranted. Nonetheless, our data raise the concern that LMB insensitive nuclear export may not always be CRM1-independent.

LMB treatment of quiescent cells prevents their subsequent progression through G1 into S phase (MC and JMS, unpublished). Since p27 export from G0 nuclei is less efficient than from nuclei in early G1, the timing of LMB addition may be important when interpreting the effects of LMB on p27 export. Using p27 immunofluorescence, others have shown that LMB blocks the transient cytoplasmic accumulation of p27 that occurs when cells are released from quiescence (Rodier *et al.*, 2001;Ishida *et al.*, 2002). If treatment with LMB in quiescence blocks G0 to G1 progression, the early G1 activation of p27 export would be compromised. Thus the lack of cytoplasmic p27 after LMB treatment observed by others may reflect failure to exit quiescence and be an indirect effect of LMB on p27 localization.

Recent data using T187A knock-in and Skp2-/- mice suggest that more than one mechanism regulate p27 proteolysis and that p27 proteolysis is T187-independent in early G1 (Malek *et al.*, 2001; Hara *et al.*, 2001). We observed that the interaction between p27 and CRM1 begins at a time in the cell cycle when both Skp2 protein levels and cyclin E-Cdk2 activities are low. Furthermore, neither nuclear export of p27 nor its binding to CRM1 are dependent on phosphorylation at T187. The present data and that of others allow the following model of two distinct mechanisms regulating p27 proteolysis. Mitogen dependent phosphorylation of p27 in early/mid-G1 may lead to a reduction in p27-cyclin E-Cdk2 binding, thereby exposing the p27 NES located within the cyclin binding domain. This would facilitate p27-CRM1 interaction and the formation of an export competent protein complex, including RanGTP. Phosphorylation at

serine 10 (S10) may be a prerequisite for subsequent events that mediate CRM1 binding and nuclear export. Both our own data and that of others indicate that S10 phosphorylation is important for CRM1 binding and required for p27 export (Rodier *et al.*, 2001; Ishida *et al.*, 2002). Binding of nucleoporins, such as Nup50, may facilitate translocation of p27 to the cytoplasm (Guan *et al.*, 2000), where it is ubiquitylated and degraded (Hara *et al.*, 2001). This early phase of export-linked p27 proteolysis appears to precede Skp2 upregulation and cyclin E-cdk2 activation and is independent of phosphorylation on T187 by cyclin E-Cdk 2, consistent with other recent reports (Malek *et al.*, 2001;Hara *et al.*, 2001;Ishida *et al.*, 2002). This initial mechanism of p27 degradation in early G1 would allow an incremental activation of cyclin E-Cdk2. This would be followed by rapid progressive kinase activation as activated cyclinE-Cdk2 mediates the further T187 phosphorylation-dependent ubiquitylation of p27 by SCF^{SKP2} and degradation in late G1 and S phase.

If an initial mechanism of titrating down p27 via nuclear export mediated degradation is required for the efficient activation of cyclin E-Cdk2 dependent p27 proteolysis, interference with export mediated p27 proteolysis could significantly alter the kinetics of G1 to S phase progression. The relevance of the subcellular localization of p27 to T187 phosphorylation dependent proteolysis remains unclear. However, it has been reported that Xic1 ubiquitylation occurs in oocyte nuclei (Swanson *et al.*, 2000) and that nuclear p27 in Rat1 fibroblasts is efficiently degraded (Rodier *et al.*, 2001). This may also be the case for p27 in epithelial cells, since both nuclear and cytoplasmic p27 levels are increased following LLnL treatment.

There appear to be multiple phosphorylation sites on p27 (Ishida *et al.*, 2000;Donovan *et al.*, 2001) whose role in regulating p27 function and degradation remain unknown. Further work is necessary to elucidate what phosphorylation events follow that of S10 in early G1 and how

p27 phosphorylation may regulate its nuclear export. While we estimate that approximately 20% of cellular p27 is present in CRM1 complexes in early to mid-G1, it remains unclear what proportion of the total cellular p27 is degraded following CRM1-mediated export. This may show both cell type variability and change during malignant tumor progression as a function of checkpoint losses that increase cyclin E-Cdk2 activity.

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Figure Legends

FIG. 1. p27 localization and CRM1-binding are cell cycle dependent. Cell cycle entry of quiescent MCF-7 cells was induced by the addition of 17β-estradiol at time 0. Cells were assayed at intervals thereafter for p27 localization (A), or for protein assays and cell cycle profiles (B). (A) MCF-7 cells grown on glass slides were arrested in G0 by estrogen depletion. Following stimulation with estradiol, p27 levels (green) and BrdU uptake (red) were visualized by confocal fluorescence microscopy at intervals across the cell cycle. Cells negative for both p27 and BrdU staining were evident in the late S phase/G2 population (white arrow). These unstained cells are apparent on phase contrast imaging of the same field. Control cells stained with non-specific control IgG followed by FITC and Texas Red conjugated antibodies are shown (IgG). (B) Transient binding of p27 to CRM1 occurs early in G1. p27 was immunoprecipitated at intervals across the cell cycle, p27 complexes were resolved and immunoblots were probed for p27 and CRM1. The same cell lysates were immunoblotted for CRM1 and Skp2. The cell cycle profile at each time point was assayed by dual PI/BrdU labeling and flow cytometry.

FIG. 2. Active nuclear export of p27. (A) Cells were grown on glass slides and synchronized as in figure 1. 6 hours after induction of cell cycle entry by 17β-estradiol addition, cells were digitonin permeabilized and subjected to export assays, fixed and nuclear p27 visualized by indirect immunofluorescence. (B) Cells were recovered in either mid-G1 or G0 and nuclear export of p27 following digitonin permeabilization assayed as described in Methods. At indicated times, reactions were stopped by centrifugation and p27 assayed in nuclei, N, and supernatant, S, fractions. p27 export was minimal after 30 minutes in the absence of ATP (-ATP), cytosol (-CYT) or both (-ATP/–CYT).

FIG. 3. p27 binds CRM1 *in vitro* and LMB does not impair p27-CRM1 binding. (A) p27 was immunoprecipitated from cells in early G1 and incubated at 95°C for 5 min to denature heat labile associated proteins. The supernatant containing p27 was then incubated with recombinant CRM1 together with either GTP-loaded Ran (lane 1) or GDP-loaded Ran (lane 2) for 30 minutes at 4°C followed by immunoprecipitation of p27. p27 antibody-bound protein A sepharose beads did not show any non-specific interaction with recombinant Ran or CRM1 (lane 3). (B) CRM1 was first immunoprecipitated from cells recovered in mid-G1 with or without prior treatment with LMB (IP# 1). The supernatant was recovered and p27 was then immunoprecipitated from the CRM1-depleted lysates (IP# 2). CRM1- and p27-bound proteins were immunoblotted for CRM1 and p27. Antibody only controls are shown for IP # 1 and # 2 (IgG). (C) The effect of LMB on binding of recombinant CRM1 to cellular p27 was assayed as in A. Equal amounts of heat stable p27 recovered from mid-G1 cells were reacted with recombinant RanGTP and CRM1 without (lane 1) or with (lane 2) pre-treatment of the CRM1 with LMB. p27 was then immunoprecipitated, complexes resolved and blotted for associated CRM1. One tenth of the input recombinant CRM1 was loaded in the lane on the right. Equal amounts of p27 were immunoprecipitated in each lane (not shown). (D) Recombinant his-tagged p27 (lanes 1-3) or flag-tagged T286-phosphorylated cyclin D1 (lanes 5-7) were incubated with RanGTP and CRM1 either without (lanes 1 and 5) or with (lanes 2 and 6) pre-treatment of the CRM1 with LMB. LMB was also added to the reaction mixture after complex formation (lanes 3 and 7). Antibody control lanes are also shown (lanes 4 and 8). (E) CRM1 binding assays were carried out as in (D). CRM1 was pre-incubated with a peptide corresponding to the NES of the HIV-1 Rev protein (NES peptide) prior to the addition of p27 (lane 2) or cyclin D1 (lane 5).

FIG. 4. Both LLnL and LMB increase nuclear and cytoplasmic p27 levels. (A) Mid-G1 cells were recovered at 9 hours after G0 release, with or without 6 hours of LLnL treatment immediately prior to harvesting. Lysates were immunoblotted for p27. (B) Cells were also treated as in A above and nuclear, N, and cytoplasmic, C, fractions immunoblotted for p27. Immunoblots were probed for the nuclear protein RCC1 to verify the lack of leakage of nuclear proteins into the cytoplasm. (C&D) At 6 hours after G0 release, cells were incubated either with or without LMB for a further 6 hours and (C) whole cell lysates or (D) nuclear and cytosolic fractions were immunoblotted as shown. RCC1 probing verified adequacy of fractionation (not shown).

FIG. 5 LMB does not prevent p27 nuclear export *in vitro* or *in vivo*. (A) MCF-7 cells were transfected with YFPp27WT and arrested in quiescence. Cells were digitonin permeabilized and incubated with 2.5 mg/ml cytosolic proteins and an ATP regenerating system. Incubations were carried out for the indicated times at room temperature. Where indicated, cells were pre-treated with LMB or LLnL to assess the affects of these drugs on p27 nuclear export. (B) The decay of nuclear p27 fluorescence in (A) was visualized by direct fluorescence microscopy, photographed with a digital camera and quantitated using Carl Zeiss laser scanning software (LSM) 510, and graphed as a function of time. (C) Hela cells transfected with expression vectors for either YFPp27 or GFPp53 were fused to non-transfected NIH 3T3 cells (white arrow) in the presence or absence of LMB. The localization of either YFPp27 or GFPp53 (green) was visualized by fluorescence microscopy. Cells were fixed and stained for actin (red). Nuclei were visualized by staining the DNA with Hoechst 33258 (black and white panels). (D) p27 nuclear import was

assessed by the addition of his-tagged p27 (His-p27) to digitonin permeabilized cells in the presence of cytosolic proteins (4 μg/μl) and an ATP regenerating system. Reactions were centrifuged and nuclear, N, and supernatant, S, fractions were immunoblotted for p27. Nuclei pre-incubated with 200 μg/ml wheat germ agglutinin (WGA) showed no import of p27.

FIG 6. p27 nuclear export involves a nuclear export sequence (A) The classical and non-classical NES of the HIV-1Rev and EIAV Rev proteins and the sequence (aa 32-45) containing the conserved leucines in the putative p27 NES from different species are shown. (B) FITC-BSA (right panel) or FITC-BSA coupled to peptides containing the putative p27 NES (2 left panes) was microinjected into the nuclei of adherant HeLa cells. Pictures at 0 min and 45 min after injection were taken with identical exposure times. (C) NES mutation delays p27 export. Cells were grown on glass slides and then transfected with YFPp27WT, YFPp27NES, YFPS10A or YFPp27T187A. Forty-eight hours after transfection, cells were digitonin permeabilized and p27 export assayed. Nuclear export of p27 was visualized directly by photomicroscopy with a digital camera. (D) The intensity of nuclear p27 fluorescence was quantitated using Carl Zeiss Laser Scanning Software (LSM) 510, and graphed as a percentage of the maximum intensity measured at t=0 minutes for each p27 allele product (p27WT, and the NES, S10A and T187A p27 mutants).

FIG. 7. Effects of p27 NES mutation on CRM1- and Cyclin E-binding (A) To show the linearity of WT p27-CRM1 binding assays, increasing amounts of heat-stable YFP-p27 (125-500 μg) were incubated with fixed amounts of CRM1 and RanGTP. p27 was immunoprecipitated and immunoblots probed for associated CRM1. (B) YFPp27 was immunoprecipitated from 300 μg of

lysate from cells transfected with WT, NES, S10A or T187A YFPp27, released from protein A beads by boiling for 10 min and incubated with recombinant CRM1 and RanGTP. p27-complexes were probed for CRM1 and p27. CRM1 binding was corrected for differences in the amount of YFPp27 expressed in the different transfectants and graphed. (C) Graphical quantification of YFP-bound CRM1. Results represent the mean ± S.E.M. of 4 independent experiments. (D) MCF-7 cells were transfected with YFPp27WT or YFPp27NES expression vectors. YFP and cyclin E immunoprecipitates were assayed for associated cyclin E, Cdk 2 or p27 by immunoblotting.

Fig 8 Effects of p27 mutation on localization and half-life (A) Cells were grown on glass slides and transfected with either WT, NES, S10A or S10D YFPp27 vectors for 16 hours and then treated with the proteasome inhibitor MG132 (+MG132) or without (-MG132) for a further 8 hours prior to fixation and photomicroscopy. (B) Cells were transfected with YFPp27WT or YFPp27NES constructs and cycloheximide (100 μg/ml) was added at 48 hours post-transfection. Cells were harvested 4, 8 and 12 hours after cycloheximide addition and p27 detected by immunoblotting with YFP-specific antibody. The decay of the p27 signal is graphed as a function of time post-cycloheximide addition. Linear regression curves were fitted to calculate the half-lives of each of the mutant p27 proteins using data from repeat experiments. Standard error bars are shown.

REFERENCES

Adachi,M., Fukuda,M., Nishida,E. (2000). Nuclear export of MAP kinase (ERK) involves a MAP kinase (MEK) dependent active transport mechanism. J.Cell Biol. *148*, 849-856.

Adam,S.A., Sterne-Marr,R., Gerace,L. (1992). Nuclear protein import using digitonin-permeabilized cells. Methods Enzymol. 97-110.

Alt,J.R., Cleveland,J.L., Hannink,M., Diehl,J.A. (2001). Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. Genes & Dev. *14*, 3102-3114.

Askjaer,P., Bachi,A., Wilm,M., Bischoff,F.R., Weeks,D.L., Ogniewski,V., Ohno,M., Niehrs,C., Kjems,J., Mattaj,I.W., Fornerod,M. (1999). RanGTPregulated interactions of CRM1 with nucleoporins and a shuttling DEADbox helicase. Mol.Cell.Biol. *19*, 6276-6285.

Askjaer,P., Jensen,T.H., Nilsson,J., Englmeier,L., Kjems,J. (1998). The specificity of the CRM1-Rev nuclear export signal interaction is mediated by RanGTP. J.Biol.Chem. *273*, 33414-33422.

Azuma,Y., Dasso,M. (2000). The role of Ran in nuclear function. Curr.Opin.Cell Biol. *12*, 302-307.

Boehm, M., Yoshimoto, T., Crook, M. F., Nallamshetty, S., True, A., Nabel, G. J., and Nabel, E. G. A growth factor-dependent nuclear kinase phosphorylates $p27^{Kip1}$ and regulates cell cycle progression. EMBO J. 21, 3390-3401. 2002.

Bogerd,H.P., Fridell,R.A., Benson,R.E., Hua,J., Cullen,B.R. (1996). Protein sequence requirements for function of the human T-cell virus type 1 Rex nuclear export signal delineated by a novel in vivo randomization-selection assay. Mol.Cell.Biol. *16*, 4207-4214.

Cariou,S., Donovan,J.C., Flanagan,W.M., Milic,A., Bhattacharya,N., Slingerland,J.M. (2000). Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells. Proc Natl Acad Sci U.S.A. *97*, 9042-9046.

Carrano,A.C., Eytan,E., Hershko,A., Pagano,M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nature Cell Biol. *1*, 193-199.

Cheng,M., Olivier,P., Diehl,J.A., Fero,M., Roussel,M.F., Roberts,J.M., Sherr,C.J. (1999). The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J. *18*, 1571-1583.

Conti,E., Kuriyan,J. (2000). Crystallographic analysis of the specific yet versatile recognition of distinct nuclear localization signals by karyopherin alpha. Structure Fold Des. *8*, 329-338.

Donovan,J.C., Milic,A., Slingerland,J.M. (2001). Constitutive MEK/MAPK activation leads to p27Kip1 deregulation and antiestrogen resistance in human breast cancer cells. J.Biol.Chem. *276*, 40888-40895.

Fornerod,M., Ohno,M., Yoshida,M., Mattaj,I.W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051-1060.

Fukuda,M., Asano,S., Nakamura,T., Adachi,M., Yoshida,M., Yanagida,M., Nishida,E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature *390*, 308-311.

Ganoth,D., Bornstein,G., Ko,T.K., Larsen,B., Tyers,M., Pagano,M., Hershko,A. (2001). The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. Nature Cell Biol. *3*, 321-324.

Gorlich,D., Kutay,U. (1999). Transport between the cell nucleus and the cytoplasm. Annual Review of Cell and Developmental Biology *15*, 607-660.

Guan,T., Kehlenbach,R.H., Schirmer,E.C., Kehlenbach,A., Fan,F., Clurman,B.E., Arnheim,N., Gerace,L. (2000). Nup50, a nucleoplasmically oriented nucleoporin with a role in nuclear protein export. Mol.Cell.Biol. *20*, 5619-5630.

Hara,T., Kamura,T., Nakayama,K., Oshikawa,K., Hatakeyama,S., Nakayama,K.I. (2001). Degradation of p27Kip1 at the G0-G1 transition mediated by a Skp2-independent ubiquitination pathway. J.Biol.Chem. *276*, 48937-48943.

Hengst,L., Dulic,V., Slingerland,J.M., Lees,E., Reed,S.I. (1994). A cell cycle-regulated inhibitor of cyclin-dependent kinases. Proc.Natl.Acad.Sci.U.S A. *91*, 5291-5295.

Hengst,L., Reed,S.I. (1996). Translational control of p27Kip1 accumulation during the cell cycle. Science *271*, 1861-1864.

Huang,T.T., Kudo,N., Yoshida,M., Miyamoto,S. (2000). A nuclear export signal in the Nterminal regulatory domain of IκBα controls cytoplasmic localization of inactive NFκB/IκBα complexes. Proc.Nat.Acad.Sci.USA *97*, 1014-1019.

Ishida,N., Hara,T., Kamura,T., Yoshida,M., Nakayama,K., Nakayama,K.I. (2002). Phosphorylation of $p27^{Kip1}$ on serine 10 is required for its binding to CRM1 and nuclear export. J.Biol.Chem. *277*, 14355-14358.

Ishida,N., Kitagawa,M., Hatakeyama,S., Nakayama,K. (2000). Phosphorylation at Serine 10, a major phosphorylation site of p27Kip1, increases its protein stability. J.Biol.Chem. *275*, 25146-25154.

Jin,P., Hardy,S., Morgan,D.O. (1998). Nuclear localization of cyclin B1 controls mitotic entry after DNA damage. J.Cell Biol. *141*, 875-885.

Kehlenbach,R.H., Dickmanns,A., Gerace,L. (1998). Nucleocytoplasmic shuttling factors including Ran and CRM1 mediate nuclear export of NFAT In vitro. J.Cell Biol. *141*, 863-874.

Kudo,N., Wolff,B., Sekimoto, T., Schreiner,E.P., Yoneda, Y., Matsumori,N., Yanagida, M., Horinouchi,S., Yoshida,M. (1998). Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. Exp. Cell Res. 242, 540-547.

Kudo,N., Matsumori,N., Taoka,H., Fujiwara,D., Schreiner,E.P., Wolff,B., Yoshida,M., Horinouchi,S. (1999). Leptomycin B inactivates CRM1/exportin by covalent modification at a cysteine residue in the central conserved region. Proc.Natl.Acad.Sci.USA. *96*, 9112-9117.

LaBaer,J., Garrett,M.D., Stevenson,L.F., Slingerland,J.M., Sandhu,C., Chou,H.S., Fattaey,A., Harlow,E. (1997). New functional activities for the p21 family of CDK inhibitors. Genes Dev. *11*, 847-862.

Macara,I.G. (1999). Nuclear transport: randy couples. Curr.Biol. *9*, R436-R439.

Malek,N.P., Sundberg,H., McGrew,S., Nakayama,K., Kyriakidis,T.R., Roberts,J.M. (2001). A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase. Nature *413*, 323-327.

Mancuso,V.A., Hope,T.J., Zhu,L., Derse,D., Phillips,T., Parslow,T.G. (1998). Posttranscriptional effector domains in the rev proteins of feline immunodeficiency virus and equine infectious anemia virus. J.Virol. *68*, 1998-2001.

Mattaj,I.W., Engimeier,L. (1998). Nucleocytoplasmic transport: the soluble phase. Annual Review of Biochemistry *67*, 265-306.

Melchior,F., Gerace,L. (1998). Two-way trafficking with Ran. Trends Cell Biol. *8*, 175-179.

Melchior, F. (1998) *Nuclear protein import in a permeabilized cell assay.* in: Protein targeting protocols (Editor R. A. Clegg) Methods in Molecular Biology *88,* 265-273.

Melchior,F., Paschal,B., Evans,J., Gerace,L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. J.Cell Biol. *123*, 1649-1659.

Millard,S.S., Yan,J.S., Nguyen,H., Pagano,M., Kiyokawa,H., Koff,A. (1997). Enhanced ribosomal association of p27(Kip1) mRNA is a mechanism contributing to accumulation during growth arrest. J.Biol.Chem. *272*, 7093-7098.

Montagnoli,A., Fiore,F., Eytan,E., Carrano,A.C., Draetta,G.F., Hershko,A., Pagano,M. (1999). Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes & Dev. *13*, 1181-1189.

Moore,M.S., Blobel,G. (1993). The GTPbinding protein Ran/TC4 is required for protein import into the nucleus. Nature *365*, 661-663.

Muller,D., Thieke,K., Burgin,A., Dickmanns,A., Eilers,M. (2000). Cyclin E mediated elimination of p27 requires its interaction with the nuclear poreassociated protein mNPAP60. EMBO J. *19*, 2168-2180.

Nigg,E.A. (1997). Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature *386*, 779-787.

Ohta,T., Michel,J.J., Schottelius,A.J., Xiong,Y. (1999). ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. Mol.Cell *3*, 535-541.

Pagano,M., Tam,S.W., Theodoras,A.M., Beer-Romero,P., Del Sal,G., Chau,V., Yew,P.R., Draetta,G.F., Rolfe,M. (1995). Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science *269*, 682-685.

Pines,J., Hunter,T. (1991). Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J.Cell Biol. *115*, 1-17.

Pines,J., Hunter,T. (1994). The differential localization of human cyclins A and B is due to a cytoplasmic retention signal in cyclin B. EMBO J. *13*, 3772-3781.

Polyak,K., Kato,J.Y., Solomon,M.J., Sherr,C.J., Massague,J., Roberts,J.M., Koff,A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. *8*, 9-22.

Reynisdottir,I., Massague,J. (1997). The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2. Genes Dev. *11*, 492-503.

Rodier,G., Montagnoli,A., DiMarticullio,L., Coulombe,P., Draetta,G., Pagano,M., Meloche,S. (2001). p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis. EMBO J. *20*, 6672-6682.

Sacer,S., Dasso,M. (2000). The ran decathlon: multiple roles of Ran. J.Cell Sci. *113*, 1111-1118.

Sheaff,R.J., Groudine,M., Gordon,M., Roberts,J.M., Clurman,B.E. (1997). Cyclin E-CDK2 is a regulator of p27Kip1. Genes & Dev. *11*, 1464-1478.

Slingerland,J., Pagano,M. (2000). Regulation of the cdk inhibitor p27 and its deregulation in cancer. J Cell Physiol. *183*, 10-17.

Slingerland,J.M., Hengst,L., Pan,C.H., Alexander,D., Stampfer,M.R., Reed,S.I. (1994). A novel inhibitor of cyclin-Cdk activity detected in Transforming Growth Factor β-arrested epithelial cells. Mol.Cell.Biol. *14*, 3683-3694.

Smitherman,M., Lee,K., Swanger,J., Kapur,R., Clurman,B.E. (2000). Characterization and targeted disruption of murine Nup50, a p27(Kip1)interacting component of the nuclear pore complex. Mol.Cell.Biol. *20*, 563156-563142.

Spruck,C., Strohmaier,H., Watson,M., Smith,A.P., Ryan,A., Krek,T.W., Reed,S.I. (2001). A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. Mol.Cell *7*, 639-650.

Stade,K., Ford,C.S., Guthrie,C., Weis,K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. Cell *90*, 1041-1050.

Stommel,J.M., Marchenko,N.D., Jimenez,G.S., Moll,U.M., Hope,T.J., Wahl,G.M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. EMBO J. *18*, 1660-1672.

Sutterluty,H., Chatelain,E., Marti,A., Wirbelauer,C., Senften,M., Muller,U., Krek,W. (1999). p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. Nat Cell Biol. *1*, 207-214.

Swanson,C., Ross,J., Jackson,P.K. (2000). Nuclear accumulation of cyclin E/Cdk2 triggers a concentration-dependent switch for the destruction of p27Xic1. Proc.Natl.Acad.Sci.USA *97*, 7796-7801.

Tomoda,K., Kubota,Y., Arata,Y., Mori,S., Maeda,M., Tanaka,T., Yoshida,M., Yoneda-Kato,N., Kato,J.Y. (2002). The cytoplasmic shuttling and subsequent degradation of p27Kip1 mediated by Jab1/CSN5 and the COP9 signalosome complex. J.Biol.Chem. *277*, 2302-2310.

Tomoda,K., Kubota,Y., Kato,J. (1999). Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1. Nature *398*, 160-165.

Tsvetkov,L.M., Yeh,K.H., Lee,S.J., Sun,H., Zhang,H. (1999). p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. Curr Biol *9*, 661-664.

Vlach,J., Hennecke,S., Amati,B. (1997). Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. EMBO J. *16*, 5334-5344.

Yang,J., Bardes,E.S., Moore,J.D., Brennan,J., Powers,M.A., Kornbluth,S. (1998). Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. Genes & Dev. *12*, 2131-2143.

Zeng,Y., Hirano,K., Hirano,M., Nishimura,J., Kanaide,H. (2000). Minimal requirements for the nuclear localization of p27Kip1, a cyclin-dependent kinase inhibitor. Biochem.Biophys.Res.Commun. *274*, 37-42.

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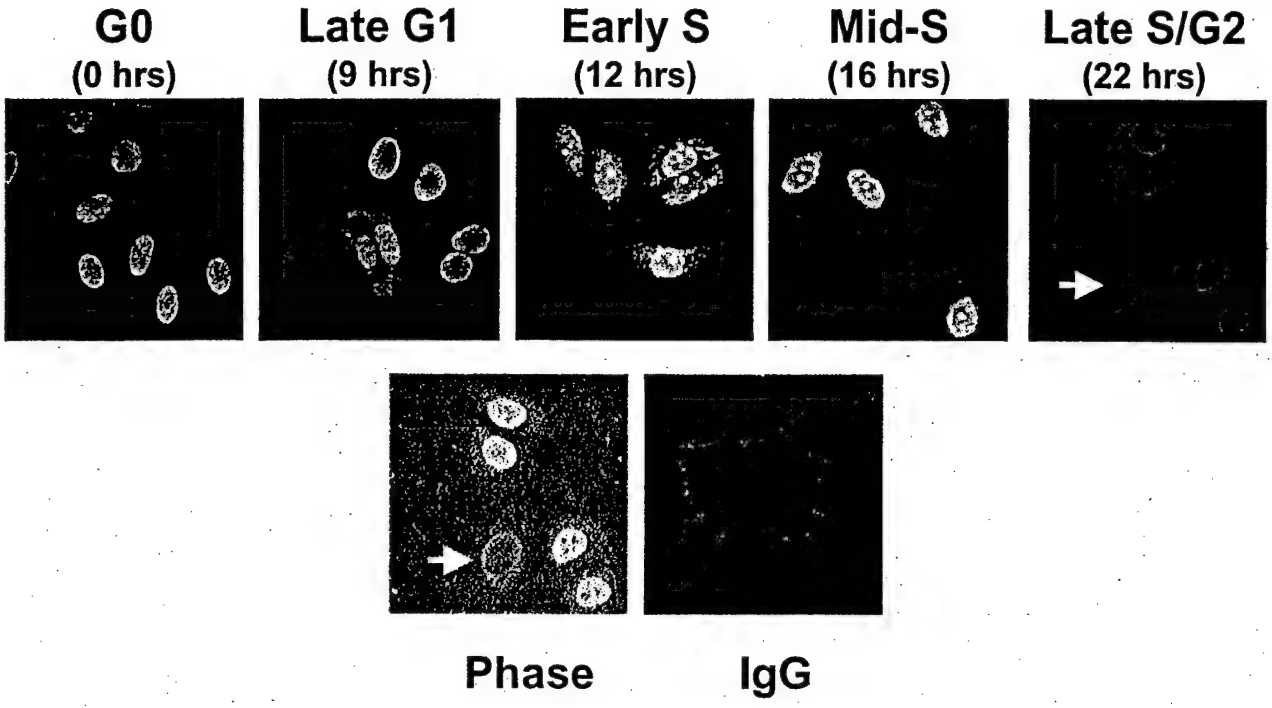

B

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G2/M	5	6	5	5	6	6	12

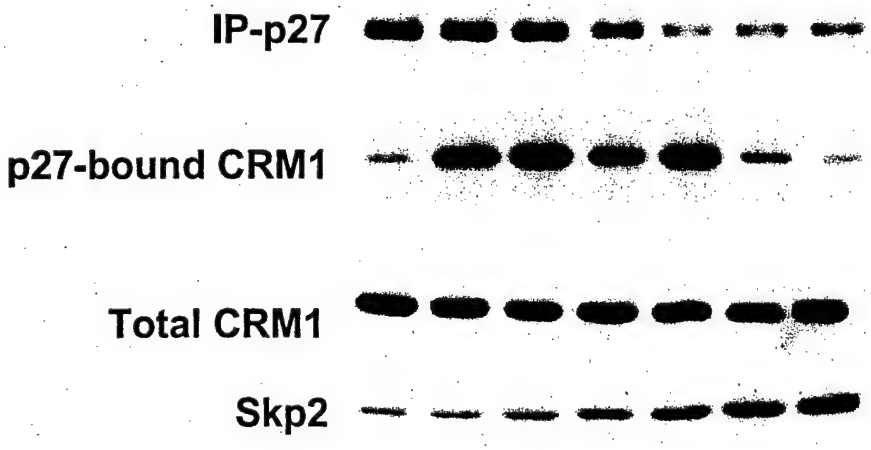

Figure 1

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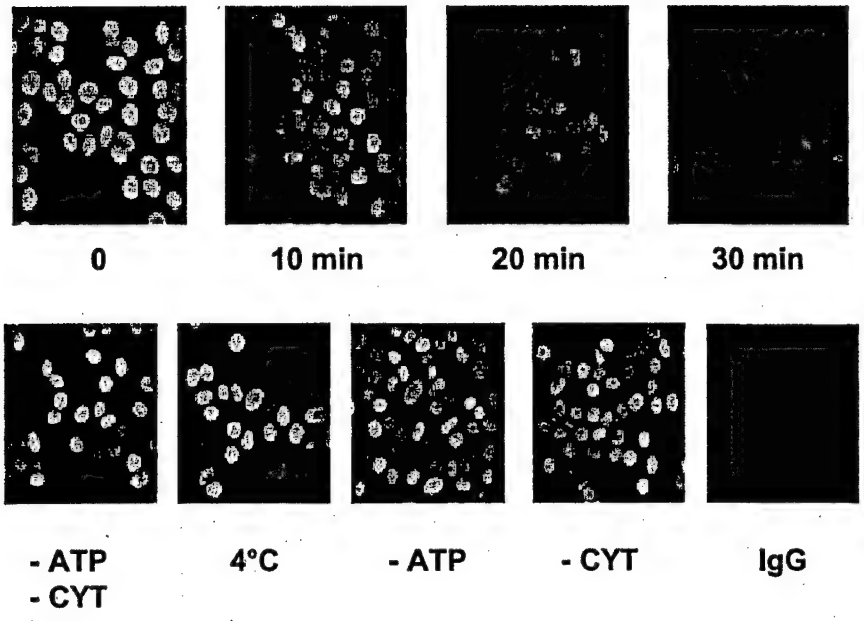
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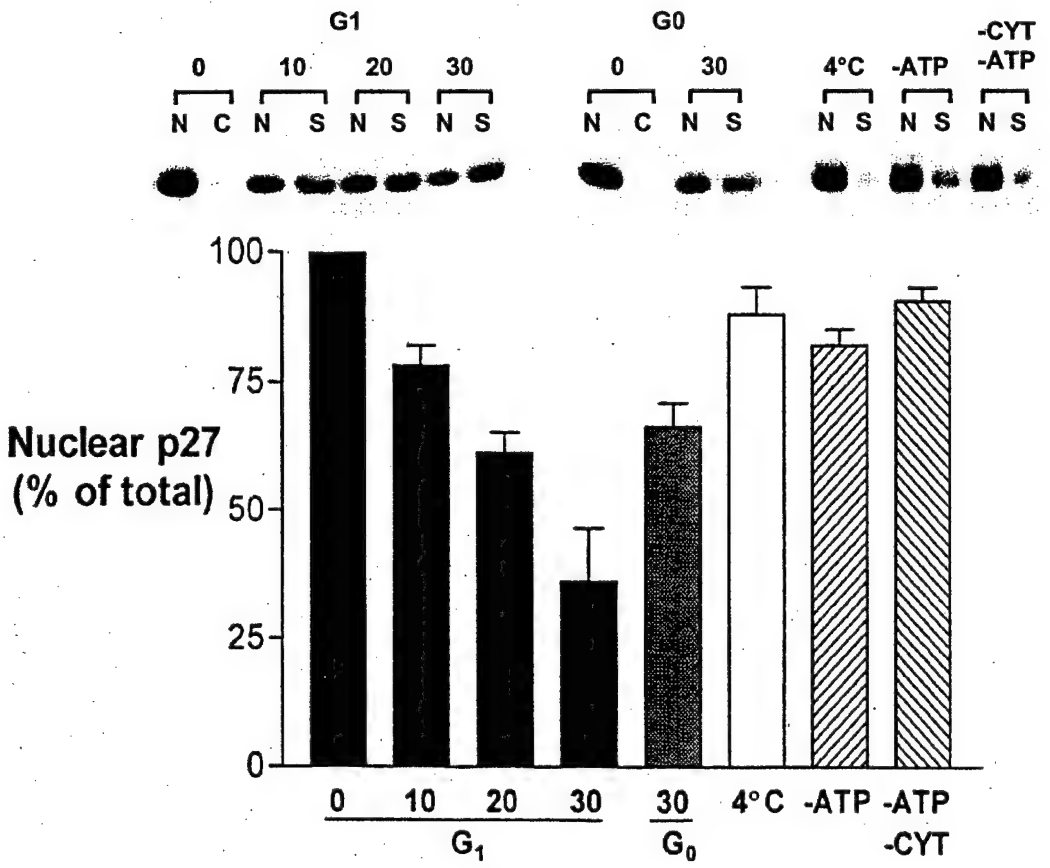
B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Nuclear p27
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Figure 2

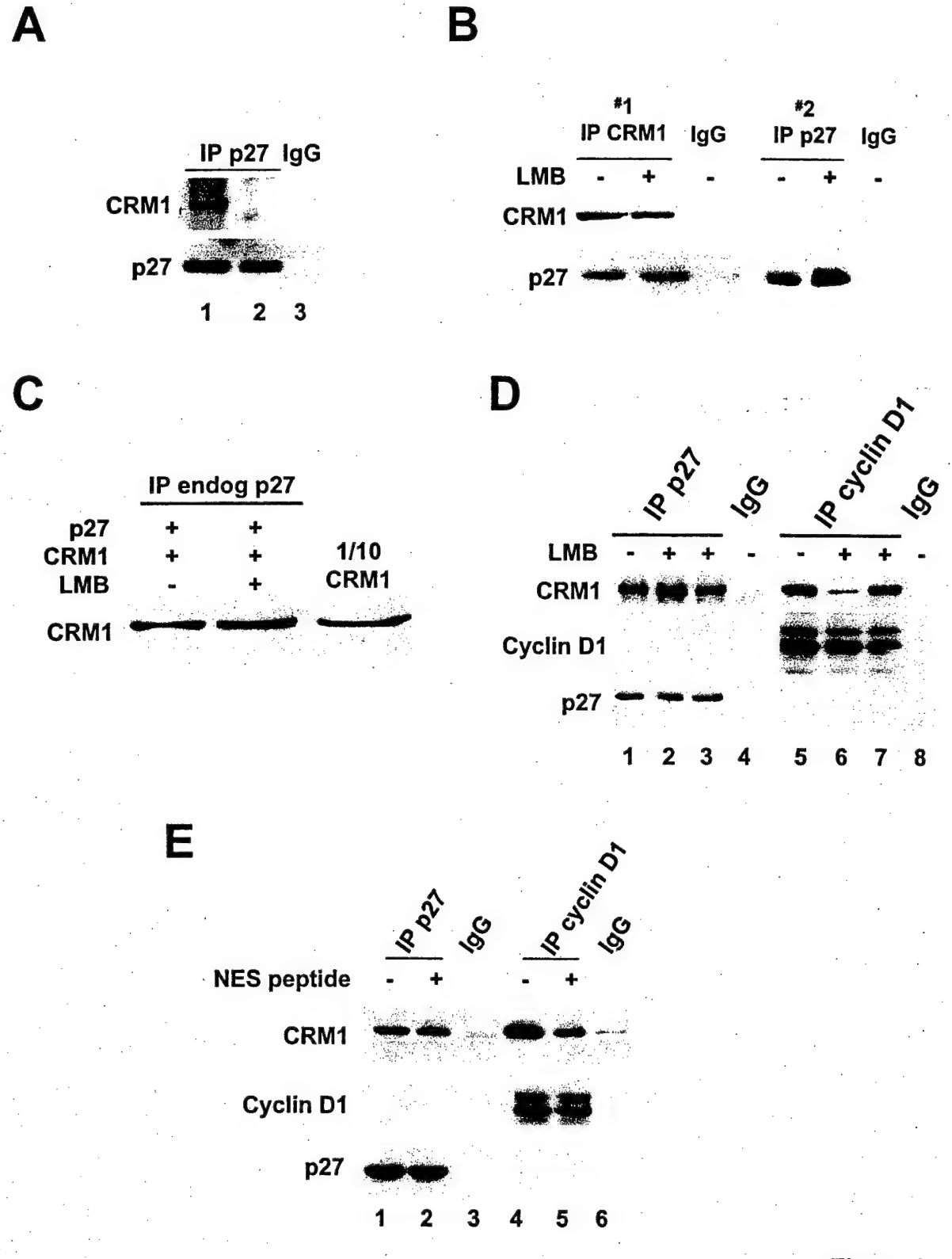
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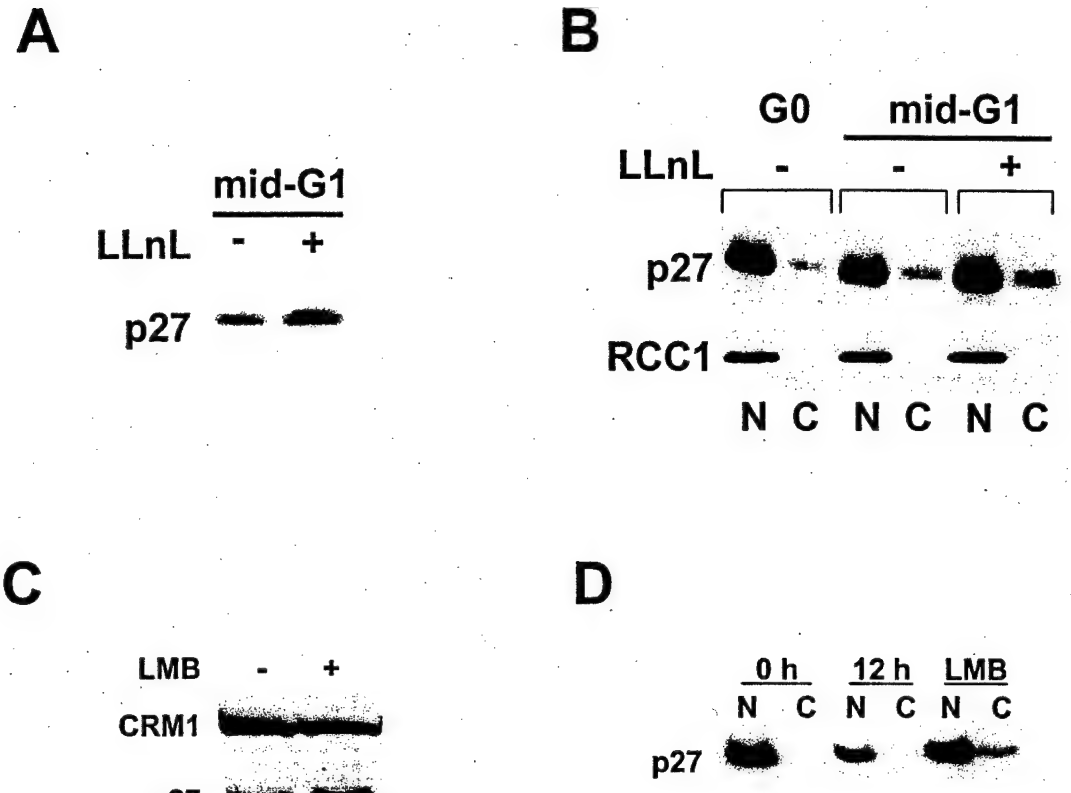
Figure 3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p27
B
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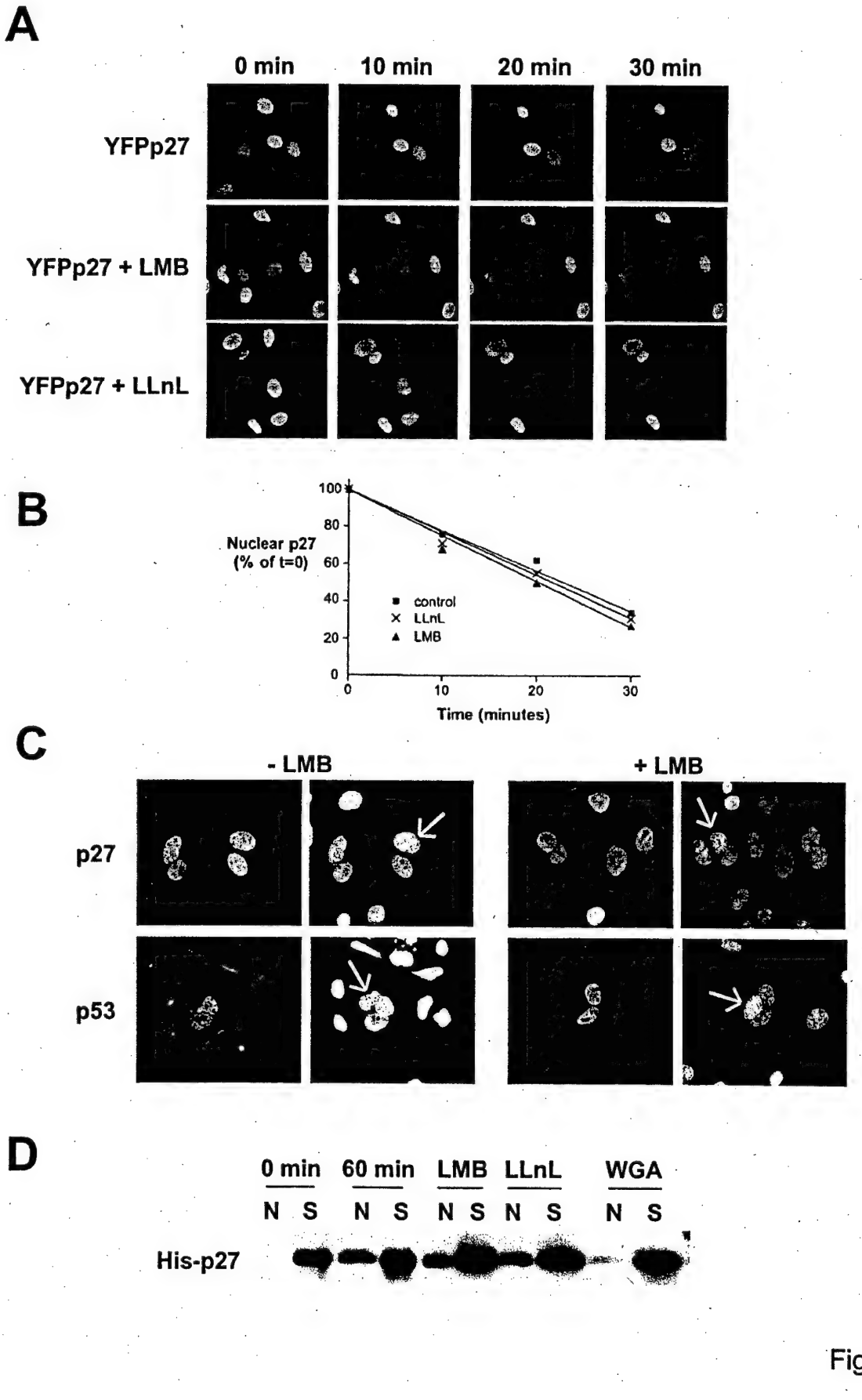
Figure 4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30 min
YFPp27
YFPp27 + LMB
YFPp27 + LLnL
B
Nuclear p27
(% of t=0)
100
80
60
40
20
0
control
LLnL
LMB
0
10
20
30
Time (minutes)
C
- LMB
+ LMB
p27
p53
D
0 min
60 min
LMB
LLnL
WGA
N S N S N S N S N S
His-p27

Figure 5

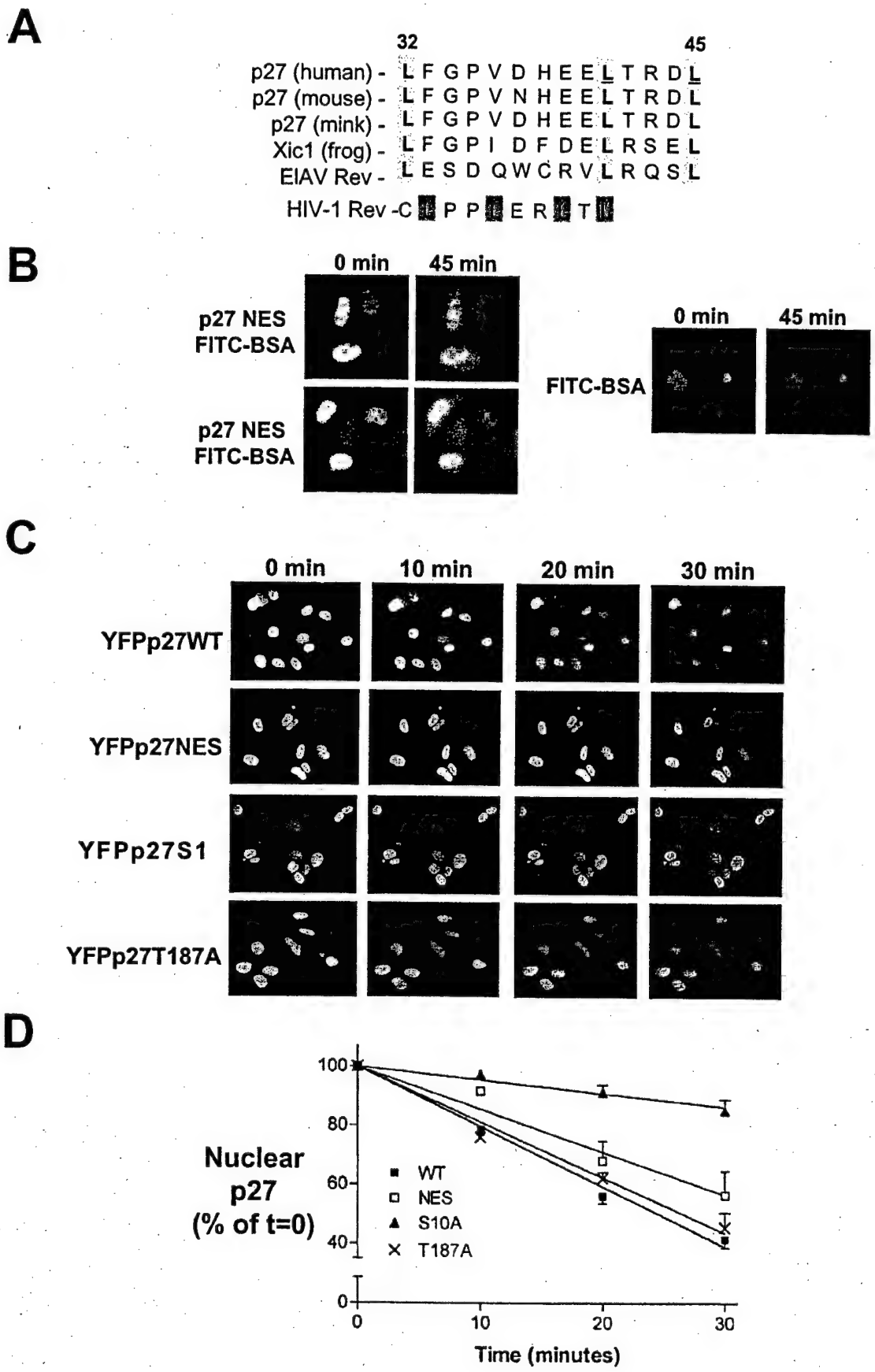

A
32
45
p27 (human) - L F G P V D H E E L T R D L
p27 (mouse) - L F G P V N H E E L T R D L
p27 (mink) - L F G P V D H E E L T R D L
Xic1 (frog) - L F G P I D F D E L R S E L
EIAV Rev - L E S D Q W C R V L R Q S L
HIV-1 Rev -C P P E R T
B
0 min
45 min
p27 NES FITC-BSA
p27 NES FITC-BSA
0 min
45 min
FITC-BSA
C
0 min
10 min
20 min
30 min
YFPp27WT
YFPp27NES
YFPp27S1
YFPp27T187A
D
Nuclear p27 (% of t=0)
100
80
60
40
0
WT
NES
S10A
T187A
0
10
20
30
Time (minutes)

Figure 6

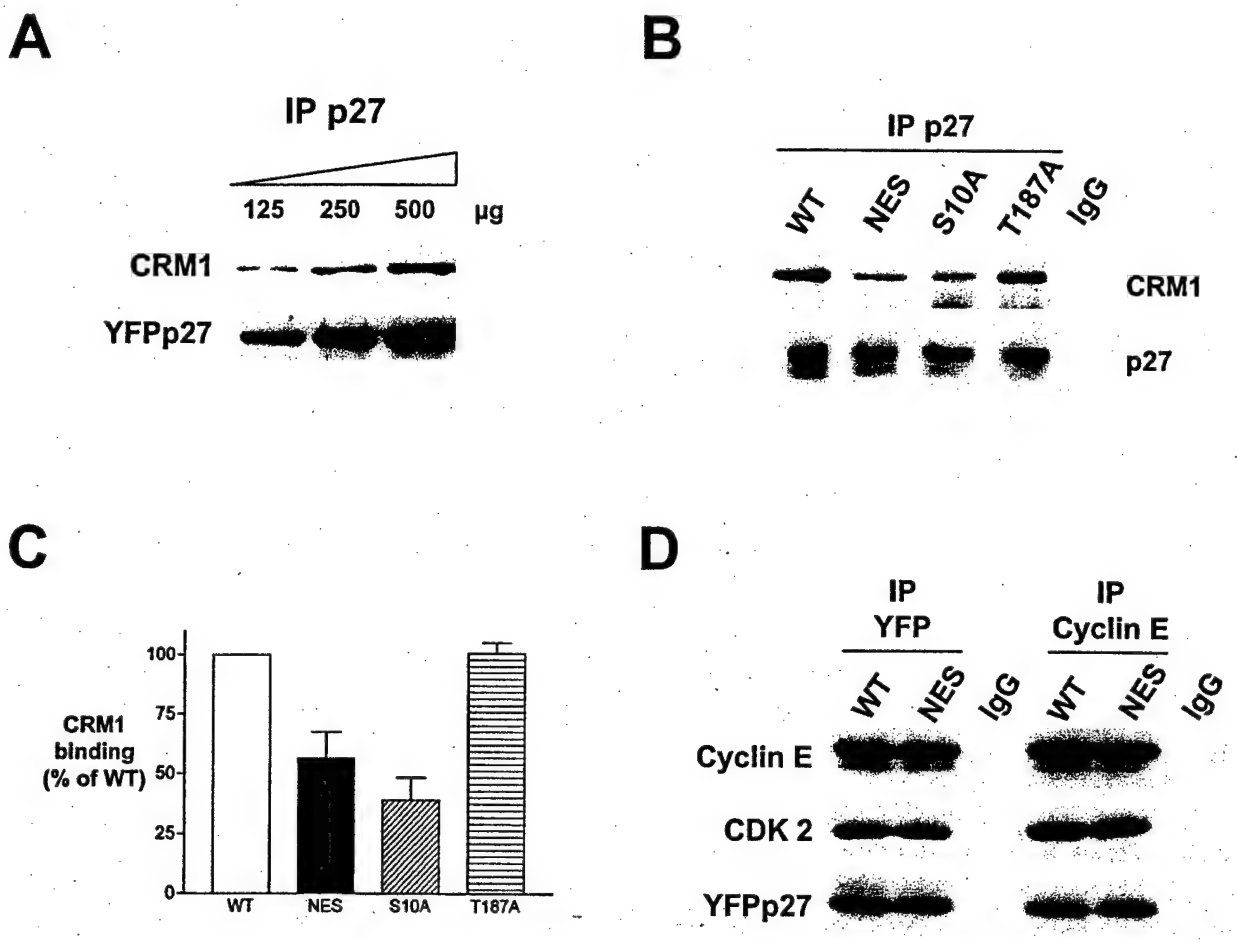
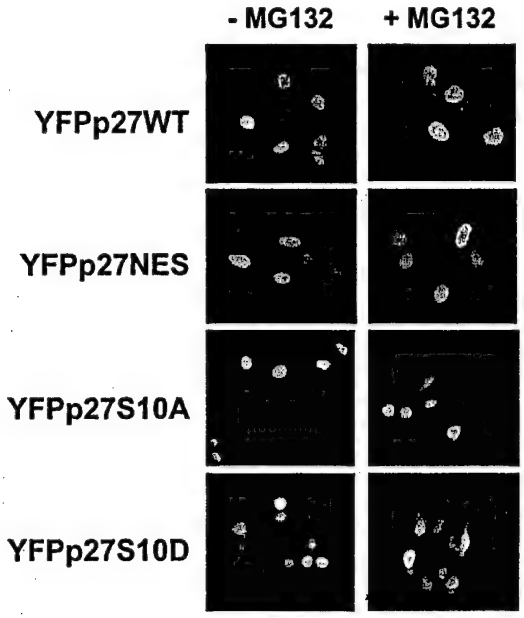

A
IP p27
125
250
500
µg
CRM1
YFPp27
B
IP p27
WT
NES
S10A
T187A
IgG
CRM1
p27
C
CRM1 binding (% of WT)
100
75
50
25
0
WT
NES
S10A
T187A
D
IP YFP
IP Cyclin E
WT
NES
IgG
WT
NES
IgG
Cyclin E
CDK 2
YFPp27

Figure 7

A


- MG132
+ MG132
YFPp27WT
YFPp27NES
YFPp27S10A
YFPp27S10D

B

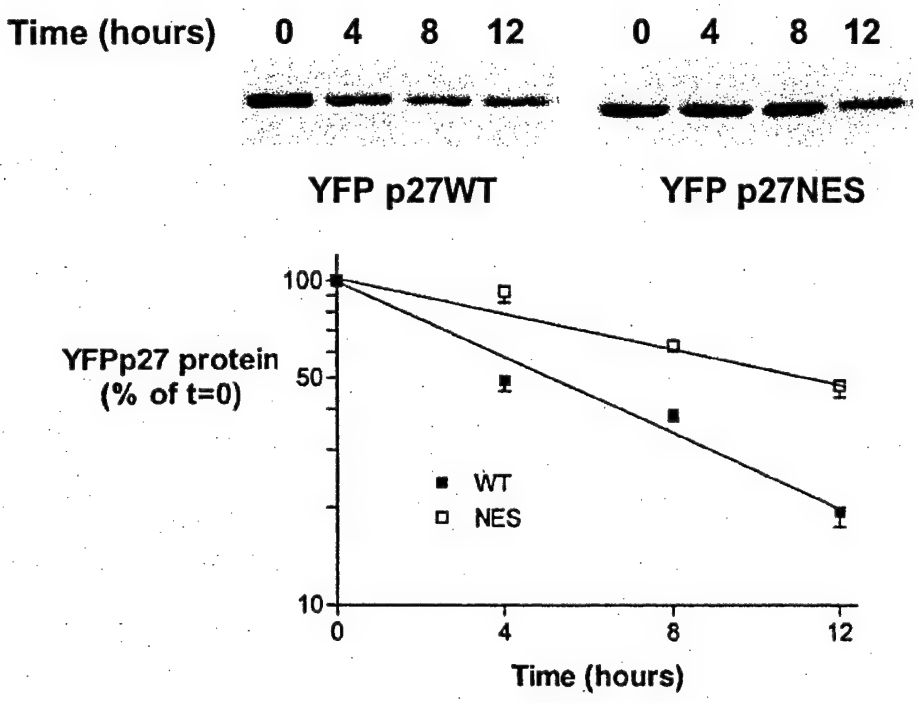

Time (hours)
0
4
8
12
0
4
8
12
YFP p27WT
YFP p27NES
YFPp27 protein
(% of t=0)
100
50
10
WT
NES
0
4
8
12
Time (hours)

Figure 8

PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest

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Mechanisms linking mitogenic and growth inhibitory cytokine signaling and the cell cycle have not been fully elucidated in either cancer or in normal cells. Here we show that activation of protein kinase B (PKB)/Akt contributes to resistance to antiproliferative signals and breast cancer progression in part by impairing the nuclear import and action of p27. Akt transfection caused cytoplasmic p27 accumulation and resistance to cytokine-mediated G1 arrest. The nuclear localization signal of p27 contains an Akt consensus site at threonine 157, and p27 phosphorylation by Akt impaired its nuclear import *in vitro*. Akt phosphorylated wild-type p27 but not p27T157A. In cells transfected with constitutively active Akt^{T308DS473D} (PKB^{DD}), p27WT mislocalized to the cytoplasm, but p27T157A was nuclear. In cells with activated Akt, p27WT failed to cause G1 arrest, while the antiproliferative effect of p27T157A was not impaired. Cytoplasmic p27 was seen in 41% (52 of 128) of primary human breast cancers in conjunction with Akt activation and was correlated with a poor patient prognosis. Thus, we show a novel mechanism whereby Akt impairs p27 function that is associated with an aggressive phenotype in human breast cancer.

Cell-cycle deregulation is a hallmark of cancer. Loss of cytokine-mediated G1 arrest may confer an advantage during malignant progression. Resistance to the antiproliferative effects of transforming growth factor-β (TGF-β) often occurs despite intact TGF-β signaling and such cells may manifest resistance to multiple inhibitory cytokines, suggesting underlying alterations in cell-cycle controls[1,2].

Cyclin-dependent kinases (cdks) are regulated by cyclin binding, phosphorylation and by two families of cdk inhibitors[3]. G1 progression is governed by D-type and E-type cyclin-cdk complexes. The inhibitors of cdk4 (INK4) family includes p15^{INK4B} and the kinase inhibitor protein (KIP) family comprises p21^{Cip1}, p27^{Kip1} and p57^{Kip2} (ref. 4). In addition to inhibition of cyclin E-cdk2, p21 and p27 also facilitate assembly and activation of cyclin D-cdks in early G1 (refs. 5,6).

p27^{Kip1} was first identified as a mediator of TGF-β1-induced G1 arrest[7–9]. TGF-β induces expression of the gene encoding *p15^{INK4B}*. p15^{INK4B} binds and inhibits cdk4 facilitating dissociation of p27 and cyclin D1 from cyclin D1–cdk4–p27 complexes and p27 binds and inhibits cyclin E-cdk2, leading to G1 arrest[10–12]. p27 also mediates G1 arrest induced by IL-6 (ref. 13). p27 is a nuclear protein whose frequent deregulation in human cancers may confer resistance to antiproliferative signals. In cMyc or MAPK activated cancer-derived lines, cyclin D1–cdk4/6 complexes sequester p27, and cyclin E-cdk2-inhibition is impaired[14–16]. cMyc inhibits p15 induction by TGF-β (ref. 17) and may also induce a factor that inactivates p27[18]. In up to 50% of human cancers, reduced p27 protein is associated with a poor prognosis[19]. In some tumors, p27 is mislocalized to the cytoplasm[19,20], however; the mechanism and significance of this has not been elucidated.

In human cancers, constitutive activation of phosphoinositol 3′ kinase (PI3K) and its effector PKB/Akt arise through oncogenic receptor tyrosine kinase activation, *Ras* activation, mutational loss of *PTEN*, or through activating mutation of the PI3K effector, protein kinase B (PKB)/Akt (hereafter termed Akt) itself[21,22]. Akt can increase cyclin D1 levels[23] and downregulate p27 by increasing p27 proteolysis[24] or repressing p27 expression through Akt phosphorylation of a forkhead transcription factor[25]. However, in most cancers, reduced p27 does not result from transcriptional silencing[19].

Here we show that Akt causes resistance to cytokine-mediated G1 arrest. p27 phosphorylation by Akt impairs its nuclear import and leads to cytoplasmic p27 accumulation. In human breast cancers, cytoplasmic mislocalization of p27 is associated with Akt activation, loss of differentiation and poor patient outcome.

Activation of Akt in lines resistant to G1 arrest by TGF-β

Ras has been shown to confer TGF-β resistance. While investigating mechanisms of TGF-β resistance, we found that two TGF-β

resistant lines showed activation of the Ras effector, PI3K-Akt pathway. TGF-β causes G1 arrest of normal 184 human mammary epithelial cells (HMECs) and of the early stage melanoma line, WM35, but not the resistant 184A1L5^R or advanced melanoma-derived line, WM239 (Fig. 1*a* and *b*). Resistant lines showed increased Akt activation (Akt-P) (Fig. 1*c* and *d*). Total Akt was similar in sensitive and resistant HMECs. Akt levels were somewhat higher in WM239 than WM35. PTEN loss contributed to Akt activation in WM239 (Fig. 1*d*).

The increased PI3K-Akt activity in 184A1L5^R and WM239 was linked to their TGF-β resistance, as partial PI3K inhibition restored TGF-β responsiveness. In 184A1L5^R, low concentrations of the PI3K inhibitor, LY294002 (10–12.5 μM) modestly reduced the S phase fraction (% S) but allowed continued proliferation, with a profile similar to that of 184 cells (Fig. 2*a*). Although TGF-β alone had little effect, 10 μM LY294002 together with TGF-β caused G1 arrest of 184A1L5^R (Fig. 2*a*). In TGF-β-resistant WM239, LY294002 at a concentration that did not independently inhibit proliferation led to G1 arrest when combined with TGF-β (data not shown).

Because inhibition of cyclin E-cdk2 by p27 contributes to G1 arrest by TGF-β, cyclin E1 complexes were assayed in 184AIL5^R. A low dose of LY294002 (10 μM) inhibited Akt activity (Fig. 2*b*). LY294002 did not change p27 protein levels in HMECs (Fig. 2*b*), but modestly increased cyclin E1–bound p27 and partly inhibited cyclin E1-cdk2 activity (Fig. 2*b*). TGF-β and LY294002 together increased further cyclin E1–bound p27 and inhibited cyclin E1-cdk2 causing G1 arrest. Thus, attenuation of P13K activity restored cyclin E1-cdk2 inhibition by p27 and G1 arrest by TGF-β.

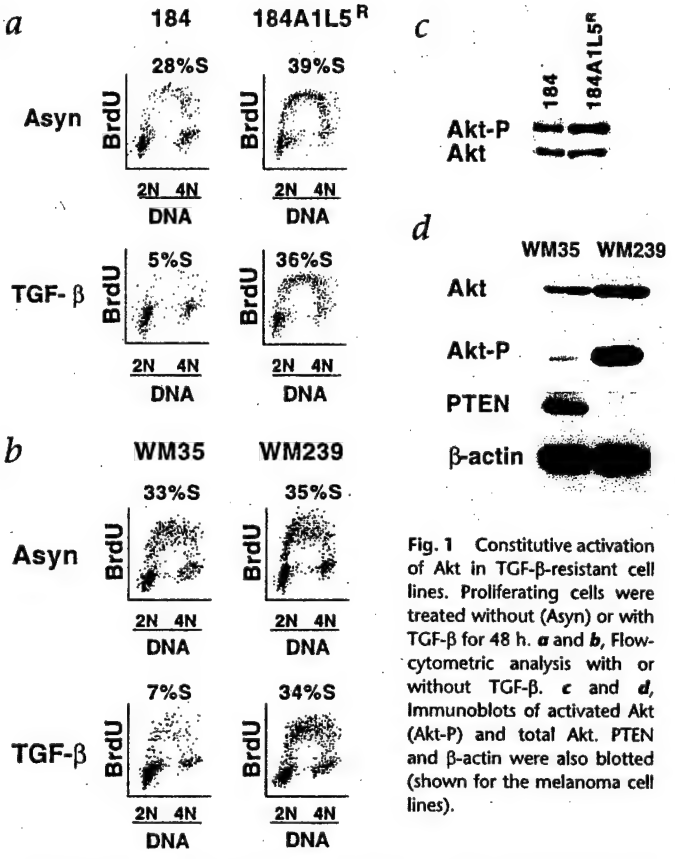

Fig. 1 Constitutive activation of Akt in TGF-β-resistant cell lines. Proliferating cells were treated without (Asyn) or with TGF-β for 48 h. *a* and *b*, Flow-cytometric analysis with or without TGF-β. *c* and *d*, Immunoblots of activated Akt (Akt-P) and total Akt. PTEN and β-actin were also blotted (shown for the melanoma cell lines).

Akt inhibits responsiveness to antiproliferative cytokines

184 and WM35 cells were transfected with a constitutively active Akt^{T308DS473D} (PKB^{DD})[26,27] or empty vector (E). Transfectants showed increased Akt protein and activity (Fig. 2*c*). PKB^{DD}-transfected lines were resistant to G1 arrest by TGF-β (shown for WM35^{PKBDD})

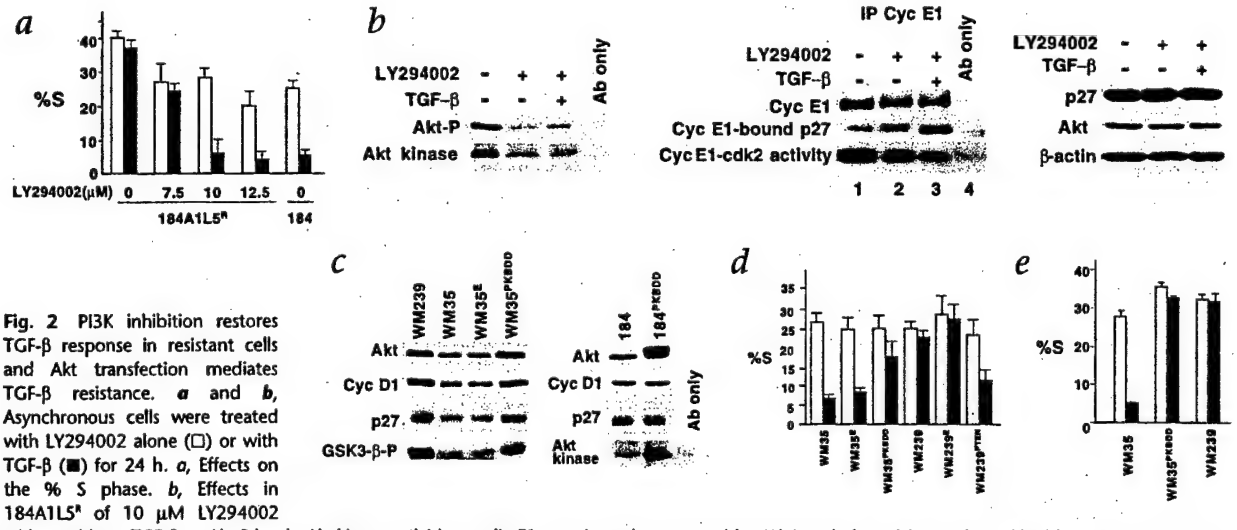

Fig. 2 PI3K inhibition restores TGF-β response in resistant cells and Akt transfection mediates TGF-β resistance. *a* and *b*, Asynchronous cells were treated with LY294002 alone (□) or with TGF-β (■) for 24 h. *a*, Effects on the % S phase. *b*, Effects in 184A1L5^R of 10 μM LY294002 with or without TGF-β on Akt-P levels, Akt kinase activities, cyclin E1-association with p27 and cyclin E-cdk2 activities. Lane 4 shows the antibody only control for the left and middle panels. Total Akt, p27 and β-actin levels are shown in the right panel. *c–e*, The indicated cells were transformed with PKB^{DD}, PTEN or empty vector (E). *c*, Ectopic expression of PKB^{DD} was shown by western blot (Akt) and Akt activity confirmed by blotting for phosphorylation of the Akt substrate, GSK3-β (GSK3-β-P) or by Akt kinase assays (Akt kinase). Levels of p27 and cyclin D1 are shown. *d* and *e*, Flow cytometric analysis of parental and transfected cells treated with (■) or without (□) TGF-β (*d*) and without (□) or with (■) IL-6 (*e*) for 48 h.

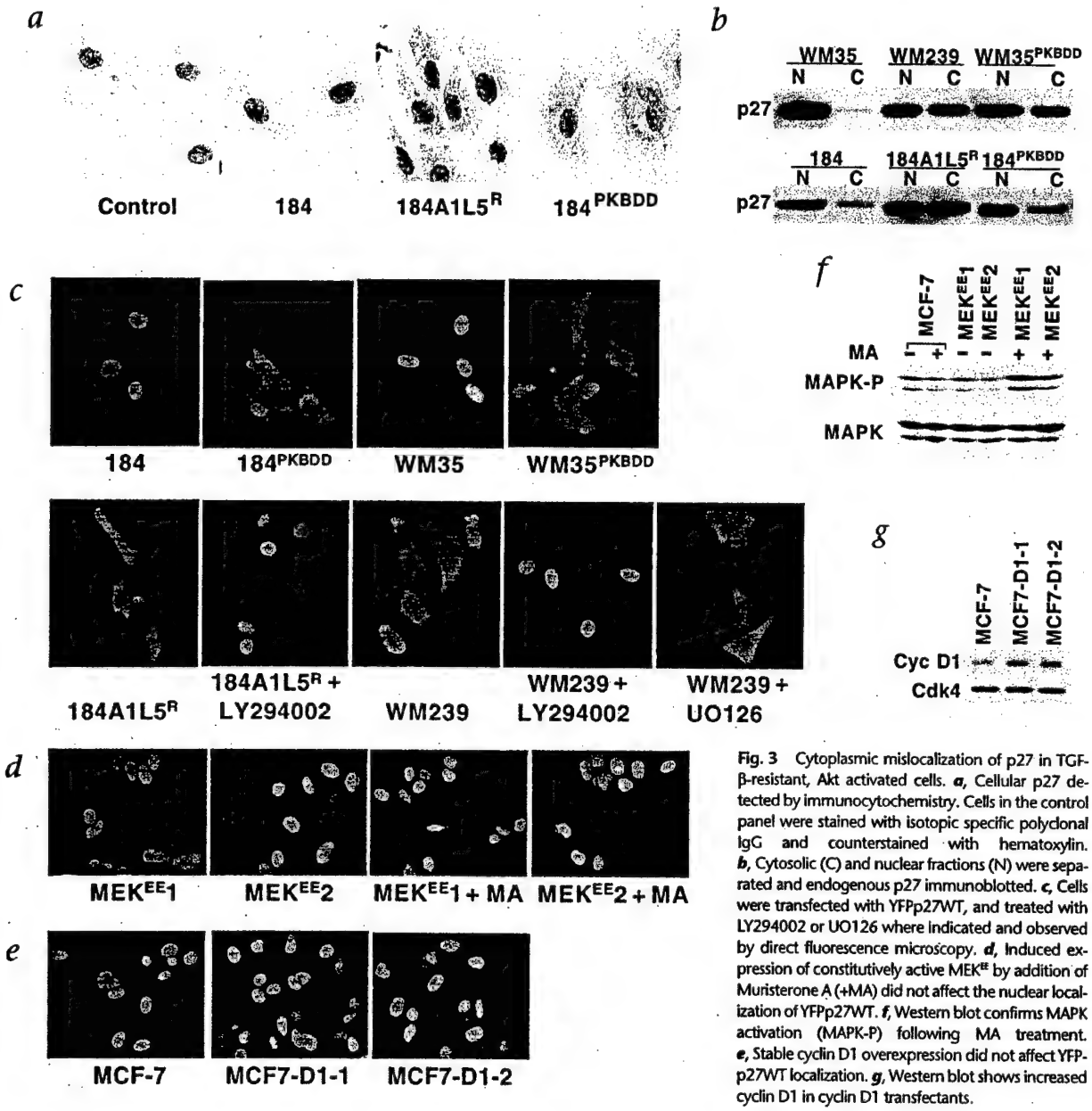

Fig. 3 Cytoplasmic mislocalization of p27 in TGF-β-resistant, Akt activated cells. ***a***, Cellular p27 detected by immunocytochemistry. Cells in the control panel were stained with isotopic specific polyclonal IgG and counterstained with hematoxylin. ***b***, Cytosolic (C) and nuclear fractions (N) were separated and endogenous p27 immunoblotted. ***c***, Cells were transfected with YFPp27WT, and treated with LY294002 or UO126 where indicated and observed by direct fluorescence microscopy. ***d***, Induced expression of constitutively active MEK^{EE} by addition of Muristerone A (+MA) did not affect the nuclear localization of YFPp27WT. ***f***, Western blot confirms MAPK activation (MAPK-P) following MA treatment. ***e***, Stable cyclin D1 overexpression did not affect YFP-p27WT localization. ***g***, Western blot shows increased cyclin D1 in cyclin D1 transfectants.

(Fig. 2*d*). Moreover, PKB^{DD} transfection also conferred resistance to G1 arrest by IL-6 (Fig. 2*e*). In WM239, transfection of PTEN restored TGF-β sensitivity (Fig. 2*d*).

Akt causes cytoplasmic mislocalization of p27

Because Akt impairs the nuclear localization of some of its substrates[28], we assayed if Akt activation affected p27 localization. Cellular p27 was predominantly nuclear in 184 HMEC. $184A1L5^R$ and 184^{PKBDD} cells showed both nuclear and cytoplasmic p27 (Fig. 3*a*). Increased cytoplasmic p27 in WM239 and $WM35^{PKBDD}$, and in $184A1L5^R$ and 184^{PKBDD} compared with WM35 and 184, respectively, was confirmed by immunoblotting of fractionated cell lysates (Fig. 3*b*).

Transfected fluorescent-tagged wild-type p27 (YFPp27WT) was exclusively nuclear in most 184 and WM35 cells (Fig. 3*c* and Supplementary Table A online). YFPp27WT-transfected $184A1L5^R$ and WM239 showed increased cytoplasmic p27. LY294002 restored the predominantly nuclear localization of YFPp27WT in TGF-β-resistant $184A1L5^R$ and WM239 cells (Fig. 3*c*). In 184^{PKBDD} and $WM35^{PKBDD}$, transfected YFPp27WT showed increased cytoplasmic localization, similar to that in $184A1L5^R$ and WM239.

In contrast to effects of LY294002, treatment of WM239 cells with the MEK inhibitor, UO126, did not result in redistribution of p27 from cytoplasm to nucleus. Moreover, in two independent cell lines, inducible overexpression of constitutively activated MEK (MEK^{EE}) and MAPK activation did not cause

cytoplasmic mislocalization of p27 (Fig. 3*d* and *f*). Thus, in this culture model, MAPK activation is neither necessary nor sufficient for cytoplasmic mislocalization of p27. As both Akt and MAPK activation can increase cyclin D1 levels, we tested the effect of cyclin D1 transfection on p27 localization. Cyclin D1 overexpression did not mediate cytoplasmic localization of p27 (Fig. 3*e* and *g*).

Akt binds and phosphorylates cellular p27^{Kip1}

A minimal consensus motif has been defined for Akt (ref. 29). p27 contains a putative Akt consensus sequence between amino acids 152 and 157 (RKRPAT). Immunoprecipitated cellular Akt could phosphorylate recombinant p27 directly *in vitro* as well as the known Akt substrate, histone H2B. p27T157A, generated by replacing the T157 with alanine, was much less efficiently phosphorylated by Akt (Fig. 4*a*). Thus, T157 is identified as a putative Akt phosphorylation site in p27. The inability of Akt to phosphorylate p27T157A is not due to loss of Akt binding as both endogenous p27 and ectopically expressed p27WT and p27T157A coprecipitated with activated cellular Akt-P (Fig. 4*b*).

To demonstrate that phosphorylation of p27 by Akt can occur in cells, p27 immunoprecipitates from asynchronous and LY294002-treated WM239 and MCF-7 cells were immunoblotted with a phospho-specific antibody against the Akt phosphorylation consensus motif (P-Akt substrate antibody). Immunoreactivity with this antibody demonstrated cellular p27 phosphorylation at the Akt consensus motif in asynchronous cells. This was inhibited by LY294002 (Fig. 4*c*).

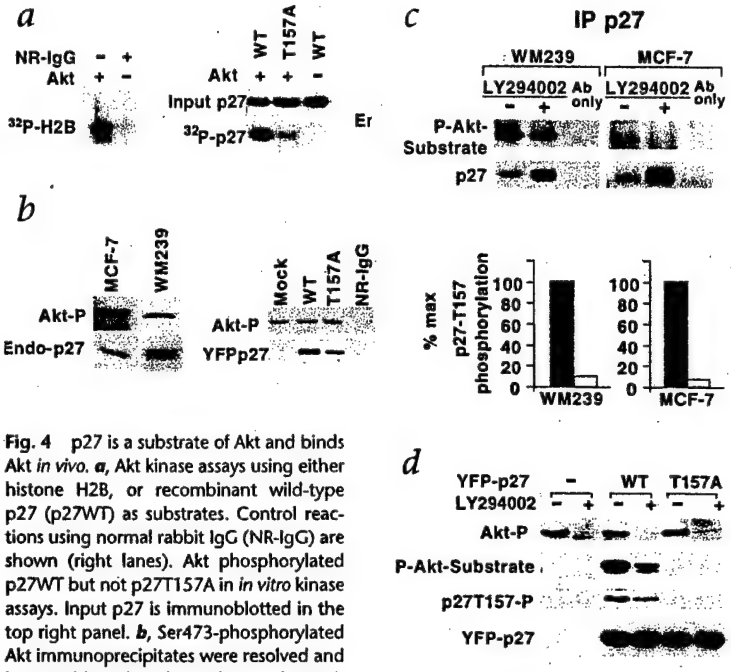

Fig. 4 p27 is a substrate of Akt and binds Akt *in vivo*. ***a***, Akt kinase assays using either histone H2B, or recombinant wild-type p27 (p27WT) as substrates. Control reactions using normal rabbit IgG (NR-IgG) are shown (right lanes). Akt phosphorylated p27WT but not p27T157A in *in vitro* kinase assays. Input p27 is immunoblotted in the top right panel. ***b***, Ser473-phosphorylated Akt immunoprecipitates were resolved and immunoblotted to show Akt-P and associated endogenous cellular p27 (left panel) or transfected YFPp27WT and YFPp27T157A (right panel). ***c***, Cellular p27 shows reactivity with antibody specific for the phosphorylated Akt consensus motif. While p27 levels are increased by LY294002, reactivity with the phospho-Akt substrate antibody (P-Akt substrate) is diminished. Lower graph: ■, without LY294002; □, with LY294002. ***d***, WM239 cells were transfected with YFPp27WT or YFPp27T157A and then treated with or without LY294002 for 20 hours. The top band shows Akt-P. YFPp27 was immunoblotted with antibodies to P-Akt substrate, anti-p27T157-P and total p27.

In normal 184 HMECs, Akt-P is minimal in G0 and increases rapidly when cells enter early G1 (data not shown). Using an antibody specific for T157-phosphorylated p27 generated by Viglietto *et al.*, we showed that cellular T157 phosphorylated p27 was minimal in G0 and the relative amount of T157 phosphorylated p27 over total p27 rose by 1.8-fold within 4 hours of exit from quiescence (data not shown). Densitometric analysis of p27T157-P and total p27 blots showed that the relative amount of T157 phosphorylated over total p27 was two-fold higher in proliferating WM239 than in WM35 (data not shown). Thus, activation of Akt *in vivo* is associated with increased T157 phosphorylation of p27 in both normal HMECs and in tumor-derived cells.

To provide further evidence for p27 phosphorylation by Akt *in vivo*, WM239 cells were transfected with either YFPp27WT or YFPp27T157A and treated with 20 μM LY294002. LY294002 markedly reduced reactivity of YFPp27WT with the antibody to the phosphorylated Akt consensus motif (P-Akt substrate) and the p27T157-P antibody. p27T157A showed minimal reactivity with the antibody to phosphorylated Akt substrate and none to anti-p27T157-P (Fig. 4*d*).

p27T157A is nuclear in cells with constitutive Akt

T157 is located within the nuclear localization signal (NLS) of p27[30,31]. Phosphorylation of the NLS regulates nuclear localization of many proteins[32]. To assay whether the potential for T157 phosphorylation might influence p27 localization, WM35^{PKBDD} cells were transfected with YFPp27WT, YFPp27T157A and YFPp27T157D (Fig. 5*a* and Supplementary Table B online). While approximately 30% of YFPp27WT and YFPp27T157D expressing cells showed both nuclear and cytoplasmic p27, YFPp27T157A was nuclear.

WM239 cells, whose Akt is constitutively activated, were transiently transfected with either YFPp27WT or YFPp27T157A. Flow cytometric analysis of YFP positive cells at 20 hours post-transfection revealed that the cell-cycle inhibitory function of p27WT was significantly impaired, while p27T157A retained G1 inhibitory function in WM239 (Fig. 5*b*). Equal expression of YFPp27WT and YFPp27T157A was demonstrated (Fig. 4*d*, lower). In contrast, YFPp27WT and YFPp27T157A both caused G1 arrest in 184 HMECs and WM35 cells lacking constitutive Akt activation (data not shown). Thus p27 phosphorylation by Akt impairs its G1 inhibitory function.

Phosphorylation by Akt impairs nuclear import of p27

p27 nuclear import was assessed by the incubation of His-tagged p27 (His-p27) with digitonin permeabilized MCF-7 cells. Nuclei and supernatant fractions were then immunoblotted for His-p27. Prior reaction of recombinant His-p27WT with cellular Akt kinase impaired nuclear import of p27. His-p27T157A showed a faster rate of import than His-p27WT, and His-p27T157D protein showed essentially no nuclear import above that of negative controls (reactions carried out at 4 °C, without ATP or in the presence of wheat germ agglutinin) (Fig. 5*c*). These data suggest that cytoplasmic p27 in Akt activated cells results from impaired p27 nuclear import.

Cytoplasmic p27 and activated Akt in human breast cancers

p27 protein levels and localization were reviewed in 128 primary breast cancers previously stained for p27 by immunohistochemistry[33]. Levels of p27 were scored as high (>50%) or low (≤50%) tumor nuclei staining as previously described[33]. While normal breast ductal epithelium and lymphocytes and a majority of tumors (Fig. 6*a*) showed exclusively nuclear p27, 42% (52/128) of the breast cancers showed either nuclear and cytoplasmic or predominantly cytoplasmic p27 (Fig. 6*b* and *c*). Of cancers with reduced p27 levels, 44% (31/70) showed cytoplasmic p27, while 36% (21/58) of tumors with high p27 levels showed cytoplasmic p27. p27 protein levels and cytoplasmic p27 mislocalization were not statistically correlated (chi square analysis, $P = 0.3542$).

Cytoplasmic p27 was not correlated with menopausal, nodal or ER/PR status. Tumors with high levels of exclusively nuclear p27 (N only; p27 >50% nuclei positive) (Fig. 6*a*) were almost all well differentiated or of low grade, whereas tumors with high levels of p27 but cytoplasmic localization (N+C; p27 >50%) (Fig. 6*b*) were more poorly differentiated ($P < 0.001$ on χ^2 analysis) (Supplementary Table C). The Kaplan–Meier curve in Fig. 6*d* shows the influence of p27 localization on patient survival. Fig. 6*e* shows that for each level of p27 staining (high, >50% nuclei positive or low, ≤50% nuclei positive), when p27 is seen in the cytoplasm (N+C) rather than in the nucleus alone (N), patient survival is worse. These data were statistically significant for overall survival ($P = 0.05$, Wilcoxon test) (Fig. 6*e*) and for disease-free survival ($P = 0.003$, data not shown). Patients with breast cancers with high levels of exclusively nuclear p27 (N only; p27 >50%) had the best outcome, whereas the worst survival was seen in those with reduced p27 levels and detectable cytoplasmic p27 (N+C; ≤50%, $P = 0.02$, Log-rank test) (Fig. 6*f*).

53 breast cancers were stained with phospho-Akt antibody (Fig. 6*a–c*, right). Normal quiescent breast acini, lymphocytes and stroma showed no Akt-P staining. None of 23 tumors with exclusively nuclear p27 showed Akt activation, whereas 28/30 tumors with cytoplasmic p27 showed phospho-Akt reactivity. Detection of cytoplasmic p27 in human breast tumors was highly statistically significantly associated with Akt activation (χ^2, $P < 0.001$).

Discussion

This study suggests that constitutive activation of the PI3K-Akt pathway mediates TGF-β resistance. TGF-β- and IL-6-sensitive cells were rendered resistant by ectopic Akt activation. Akt activity was increased in 184A1L5^R and WM239 cells. Moreover, *PTEN* transfection into WM239 cells, and inhibition of PI3K by LY294002 in TGF-β-resistant cells restored p27 binding and inhibition of cyclin E-cdk2 and G1 arrest by TGF-β.

Whereas PI3K signaling can inhibit p27 transcription or accelerate p27 degradation in different cell types, we show that Akt can bind p27 and phosphorylate T157. Akt phosphorylated p27WT but not p27T157A *in vitro*. Moreover, the reactivity of cellular and transfected p27 with phospho-Akt substrate and p27T157-P antibodies was strongly reduced by Akt inhibition following LY294002 treatment. The p27T157A showed minimal reactivity with the antibody to phosphorylated Akt substrate and none with anti-p27T157-P. Cellular p27 phosphorylation at T157 increased in normal HMECs in association with Akt activation as cells moved from G0 into G1. Moreover, T157 phosphorylation of p27 was two-fold higher in PTEN-deficient WM239 than in WM35 cells, indicating that T157 of p27 is a putative Akt site *in vivo*.

T157 lies within the nuclear localization signal of p27 (NLS, aa 153–166)[30,31]. Our data indicate that an Akt-dependent pathway regulates p27 localization. Although the T157A mutation may facilitate p27 import irrespective of PKB, phosphorylation at this site appears to inhibit nuclear import of p27. Cells with activated Akt showed cytoplasmic p27. LY294002 treatment or expression of p27T157A in cells with activated Akt restored nuclear p27 localization. Moreover, Akt^{DD} transfection led to cytoplasmic mislocalization of p27. p27 phosphorylation by Akt impaired nuclear p27 import *in vitro*. Phosphorylation within or near the NLS has been shown to inhibit nuclear import of other proteins[32]. Thus, as for other Akt substrates p21, and some forkhead transcription factors (refs. 28,34), in cancers the constitutive p27 phosphorylation by Akt may cause a relative cytoplasmic sequestration of p27. This would limit the p27 available to bind to and inhibit cyclin E-cdk2, compromising the arrest response to antiproliferative factors such as TGF-β and IL-6. In contrast, in normal HMECs, T157 phosphorylation of p27 ac-

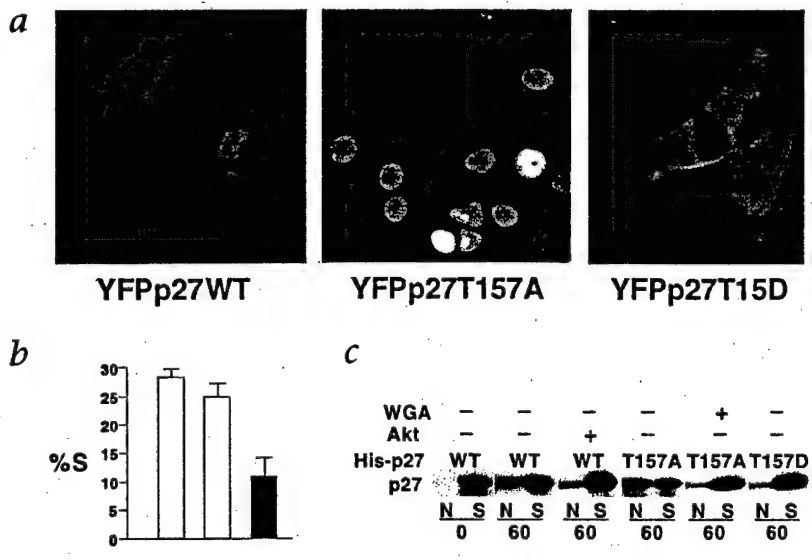

Fig. 5 T157 phosphorylation impairs nuclear import of p27. ***a***, p27 localization following transfection of the indicated p27 alleles into WM35 cells expressing PKB^{DD} (WM35^{PKBDD}). ***b***, WM239 cells were transiently transfected with YFPp27 alleles and the cell-cycle profile of YFP-positive cells analyzed by flow cytometry. □, untransfected; ▩, wild-type; ■, T157A-transfected. ***c***, p27 nuclear import was assessed by the addition of His-tagged p27 (His-p27) to digitonin permeabilized cells for 60 minutes (60) and nuclear, (N), and supernatant, (S), fractions were immunoblotted for p27. Input His-p27WT is shown in the left-most lane (t=0 minutes). Pre-treatment of His-p27WT with cellular Akt (+Akt) impaired p27 import. Import reactions for His-p27T157A (T157A) and His-p27T157D (T157D) are shown. Import of T157A was abolished by addition of wheat germ agglutinin (+ WGA).

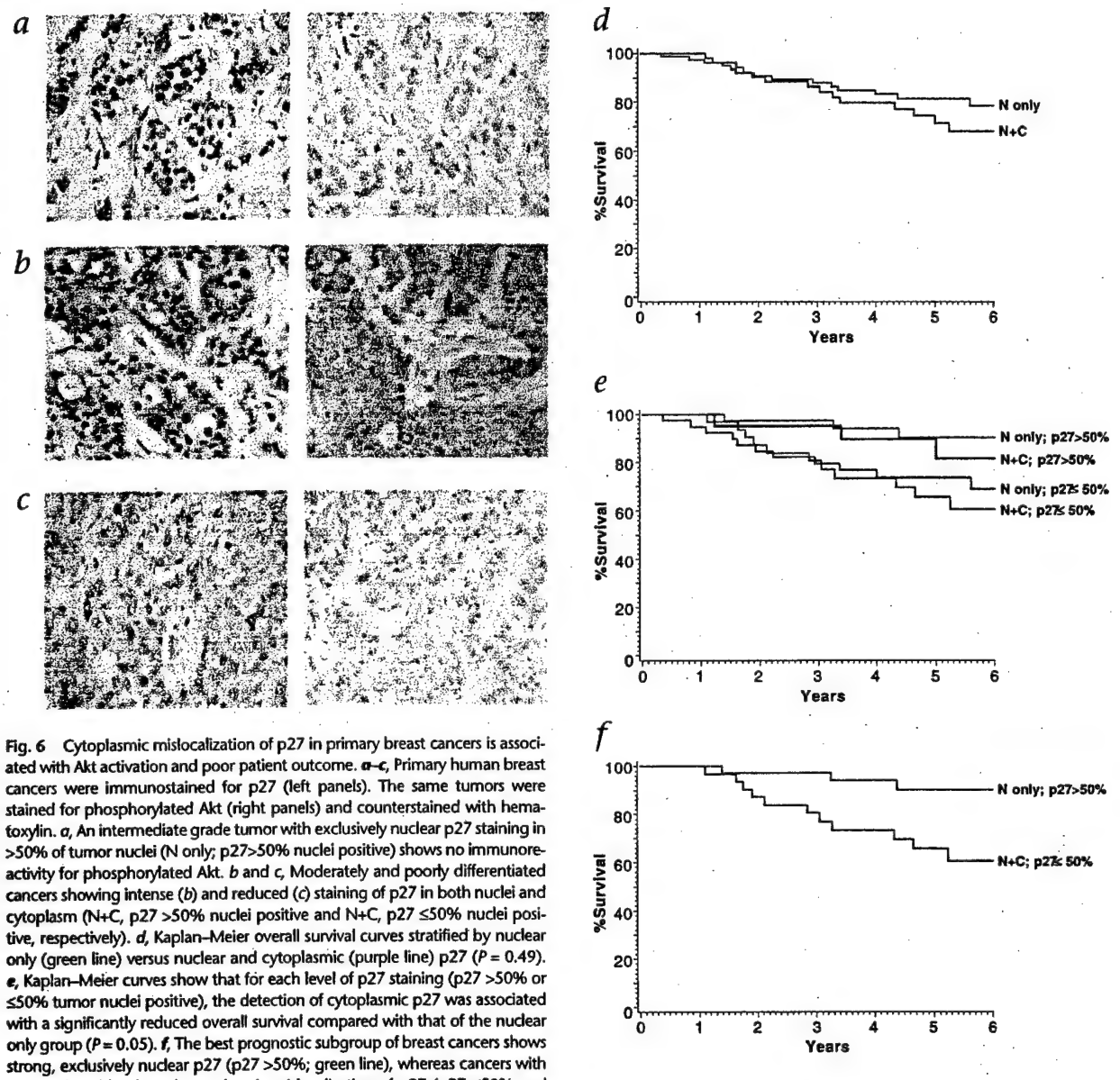

Fig. 6 Cytoplasmic mislocalization of p27 in primary breast cancers is associated with Akt activation and poor patient outcome. *a–c*, Primary human breast cancers were immunostained for p27 (left panels). The same tumors were stained for phosphorylated Akt (right panels) and counterstained with hematoxylin. *a*, An intermediate grade tumor with exclusively nuclear p27 staining in >50% of tumor nuclei (N only; p27>50% nuclei positive) shows no immunoreactivity for phosphorylated Akt. *b* and *c*, Moderately and poorly differentiated cancers showing intense (*b*) and reduced (*c*) staining of p27 in both nuclei and cytoplasm (N+C, p27 >50% nuclei positive and N+C, p27 ≤50% nuclei positive, respectively). *d*, Kaplan–Meier overall survival curves stratified by nuclear only (green line) versus nuclear and cytoplasmic (purple line) p27 ($P = 0.49$). *e*, Kaplan–Meier curves show that for each level of p27 staining (p27 >50% or ≤50% tumor nuclei positive), the detection of cytoplasmic p27 was associated with a significantly reduced overall survival compared with that of the nuclear only group ($P = 0.05$). *f*, The best prognostic subgroup of breast cancers shows strong, exclusively nuclear p27 (p27 >50%; green line), whereas cancers with both reduced levels and cytoplasmic mislocalization of p27 (p27 ≤50%; red line) have the lowest survival ($P = 0.02$).

companies the periodic activation of Akt and may regulate normal p27 function in early G1. It is noteworthy that the PKB consensus sequence in p27 is imperfect and shows some species variation. While PKB may phosphorylate p27 at this site in humans, the possibility that other kinases phosphorylate T157 cannot be excluded.

PKB^{DD} transfection increased cyclin D1 levels in the $WM35^{PKBDD}$ but not in 184^{PKBDD}, but p27 was mislocalized to cytoplasm in both cell types. PKB^{DD} mediated cytoplasmic p27 mislocalization does not result from increased cyclin D1, since cyclin D1 overexpression did not cause cytoplasmic sequestration of p27 in our assays.

Whereas many reports have shown the importance of accelerated p27 proteolysis causing reduced p27 in human cancer[19], only one study showed an effect of cytoplasmic p27 on outcome in esophageal cancer[20]. Patient data in which p27 loss and p27 localization are analyzed together with respect to disease outcome have not been published to date. Here, we demonstrate that Akt-mediated phosphorylation of p27 in its NLS impairs its nuclear import *in vitro* and show that cytoplasmic p27 localization is linked to Akt activation in human breast cancer *in vivo* and is associated with reduced patient survival.

Three independent reports (including this study) demonstrate cytoplasmic p27 in up to 40% of primary human breast cancers in association with activated Akt (Viglietto *et al.* and Shin *et al.* in this issue). Serine 473-phosphorylated Akt was not detected in normal breast epithelium and Akt-P staining showed greater intensity in invasive than in non-invasive tumor areas within individual breast cancers (unpublished data). Tumors with uniquely

nuclear p27 localization showed no Akt activation.

Although Akt can inhibit p27 gene expression by targeting the forkhead transcription factors[25] and loss of *PTEN* may lead to accelerated p27 proteolysis[24], this seems to be cell-type dependent and may require additional changes in signaling pathways. LY294002 increased p27 protein in the melanoma lines but not in normal HMECs. In HMECs and in human breast cancers, Akt activation was not always associated with p27 loss. There was no statistical correlation between cytoplasmic p27 and reduced p27 protein in primary breast cancers. Approximately the same proportion of tumors with high and low p27 scores showed cytoplasmic p27. One possible implication of this is that the processes leading to cytoplasmic p27 mislocalization and accelerated p27 proteolysis may arise independently *in vivo* and each may contribute to tumor progression.

For all levels of p27 staining, the presence of p27 in the tumor-cell cytoplasm was associated with reduced differentiation and lower disease-free survival ($P = 0.003$) and overall survival ($P = 0.05$). These data have implications for the clinical application of p27 as a prognostic factor. Taking into account the presence or absence of cytoplasmic p27 may add to the prognostic significance of reduced p27 levels. Larger studies will be needed to confirm this.

Although one report suggested that p21 phosphorylation by Akt leads to its cytoplasmic sequestration[34], two other groups confirmed that p21 is a Akt substrate, but did not observe cytoplasmic mislocalization of p21 upon Akt activation[35,36]. p21 is expressed more sporadically and at lower levels than p27 in human breast cancers and previous studies have shown conflicting results regarding the prognostic potential of p21 (ref. 37). Although mislocalization of both p21 and p27 could theoretically cooperate to promote tumor progression and a comprehensive study of p21 and p27 levels and localization would be of value, p27 may have greater potential clinical utility in cancer prognosis.

Recent reports suggest that MAPK activation accelerates p27 proteolysis[38,39]. This study, together with reports from Viglietto and Arteaga, suggest that constitutive PI3K-Akt activation contributes to oncogenesis through inhibition of nuclear p27 import and hence its cdk inhibitory function. Cytoplasmic mislocalization of p27 worsens the prognosis associated with reduced p27 levels in breast cancer supporting the relevance of these mechanisms to human tumorigenesis. Although some tumors show evidence of both accelerated p27 proteolysis and cytoplasmic localization, others show only one or the other. In human cancers, mutational activation of *Ras* and loss of the tumor suppressor *PTEN* are not infrequent[21,22]. Overexpression of receptor tyrosine kinases (RTKs), such as Her2, can also activate PI3K-Akt in human breast and other cancers[40]. As both PI3K and MAPK are downstream of RTK-Ras, it will be of interest to determine what additional pathways direct RTK signaling to mediate either p27 proteolysis or cytoplasmic mislocalization in some breast cancers, while in others both coexist.

Akt phosphorylates proteins involved in signal transduction, apoptosis and gene expression[40], and increasing evidence suggests that this pathway contributes importantly to cell-cycle regulation. Although this pathway affects multiple cell-cycle effectors, including cyclin D and p21, the present study, together with those of Arteaga and Viglietto, define a novel mechanism linking Akt activation with impaired nuclear p27 import and p27 deregulation in human cancer.

Methods

Cell culture. Finite life span HMEC strain 184 (ref. 41) and immortalized 184AIL5[R] (ref. 12), MCF-7 cells[42] and WM35 and WM239 lines[43] were cultured as described. Cells were treated with 10 ng/ml TGF-β.

Plasmids, site-directed mutagenesis and transfection. The retroviral pBABE vector or pBABE constructs carrying the constitutively activated, hemagglutinin (HA)-tagged $PKB^{T308D/S473D}$ (PKB^{DD})[26,27] or wild-type PTEN were transfected as described[26]. Human wild-type p27 cDNA (p27WT) was inserted into the pIND vector. T157 of p27 was replaced with alanine (p27T157A) or aspartic acid (p27T157D) by site-directed mutagenesis using a QuickChange site-directed mutagenesis kit (Strategene, Loyola, California). The WT and mutant p27 cDNAs were then subcloned into pEYFP-C1 vector (Clontech, Palo Alto, California) encoding an N-terminal yellow-green variant of the *Aequorea victoria* green fluorescent protein (YFP). The YFP-p27 vectors were transfected using LipofectAMINE/PLUS reagents (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. Muristerone A (MA) inducible MEK^{EE} expression was achieved in MCF-7 cells using the 2-plasmid system from Invitrogen. In the absence of MA, cells showed basal MAPK activity. MA increased MAPK-P in 2 different MEK^{EE} inducible lines. pRC-CMV-cyclin D1 (provided by P. Hinds) was transfected into MCF-7 cells to generate cyclin D1–overexpressing cell lines.

Intracellular localization of p27. HMECs were EGF-depleted for 48 h and endogenous p27 detected by immunocytochemistry as described[33]. Nuclear cytoplasmic fractionation was carried out by digitonin-permeabilization as described[44]. Nuclei were isolated by centrifugation and the supernatant containing the cytosolic fraction collected. Equal cell volumes of nuclear and cytoplasmic lysates were assayed for p27 by immunoblotting. The nuclear protein, RCC1, was blotted as a fractionation control. YFP-tagged p27 expression was detected by direct fluorescence microscopy of transfected cells. Photographs were taken at ×400 magnification using an Empix digital camera and 'CoolSnap' (Photomajics, Pittsfield, Massachusetts) software.

Nuclear import assays. Import assays were carried out as described[44]. MCF-7 cells were digitonin permeabilized and isolated nuclei incubated with cytosolic proteins (4 μg/μl), an ATP-regenerating system and recombinant his-tagged WT, T157A or T157D p27 for 60 min at 21 °C. Nuclear and supernatant fractions were then separated by centrifugation and immunoblotted for His-p27. Where indicated, His-p27WT was reacted *in vitro* with cellular Akt before import assays. Pre-incubation with wheat germ agglutinin (200 μg/ml) abolished active p27 import.

Flow cytometry. Cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdU), stained with anti-BrdU-conjugated FITC (Becton Dickinson, Mountain View, California) and propidium iodide and cell-cycle data acquired as described[12].

Recombinant protein, affinity purification and kinase assays. The p27WT, p27T157A and p27T157D cDNAs were subcloned into pET28a. Recombinant His-p27 was purified on nickel-agarose beads. An antibody against serine 473 (Ser473) phosphorylated Akt (ref. 26) was used to assay Akt activation by immunoblotting. For assays of Akt activity, Akt was immunoprecipitated from 1 mg cell lysates and reacted with GSK-3-α as substrate using an Akt kinase kit (New England Biolabs, Beverly, Massachusetts). Akt kinase assays were also carried out using either 5 μg histone-H2B or 10 μg recombinant His-p27 as substrates as described[45]. Cyclin E1-dependent kinase activity was assayed and quantified as described[9].

Antibodies and immunoblotting. Antibodies to Akt, GSK-3-β, phospho-Akt, phospho-GSK3-β (Thr21) were obtained from New England Biolabs; to p27 (C-19) and cdk2 from Santa Cruz Biotechnology (San Cruz, California); to p27 (DCS72) from Neomarkers (Fremont, California); to p27 from Transduction Labs (Lexington, Kentucky); to β-actin from Sigma (Oakville, Ontario). Cyclin E1 antibodies (mAbs E12 and E172) were obtained from E. Harlow. Antiphospho-Akt-substrate antibody was from New England Biolabs. The antibody specific for T157-phosphorylated p27 was generated and provided by G. Viglietto. Cells were lysed in ice-cold NP-40 lysis buffer and immunoblotted as described[12].

Patient population and statistical methods. The population studied was a group of 128 patients who underwent surgery for non-metastatic primary breast cancer between 1986 and 1992 at the Sunnybrook Health Sciences Center. This study was approved by the Research Ethics Board of the hospital. Kaplan-Meier survival and disease free survival curves were generated using nuclear p27 score and nuclear versus nuclear and cytoplasmic p27 as strata. Log-rank, Wilcoxon and –2log (LR) were used to assess significance. The association between discrete variables was tested using the χ^2 test.

Immunohistochemistry. Paraffin sections of tumor blocks were stained for p27 as described[33] using monoclonal p27 antibody (Transduction Labs, Lexington, Kentucky) diluted 1:1000 (0.25 μg/ml) in PBS or for phospho-Akt using the phospho-Akt (Ser473) antibody (NEB) diluted 1:200. Sections were counterstained with hematoxylin. The degree and localization of p27 staining was scored independently by two pathologists (C.C. and J.Z.) and J.M.S. as described[33]. Tumors showing both nuclear and cytoplasmic or exclusively cytoplasmic p27 in at least 35% of cells were scored 'N+C'. Tumors with exclusively nuclear p27 were scored as nuclear only (N only). Phospho-Akt was scored by J.Z. and J.M.S.

Note: Supplementary information is available on the Nature Medicine website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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1. Donovan, J. & Slingerland, J. Transforming growth factor-β and breast cancer: Cell cycle arrest by transforming growth factor-β and its disruption in cancer. *Breast Cancer Res.* **2**, 116–124 (2000).
2. Kretzschmar, M. Transforming growth factor-β and breast cancer: Transforming growth factor-β/SMAD signaling defects and cancer. *Breast Cancer Res.* **2**, 107–115 (2000).
3. Sherr, C.J. G1 phase progression: Cycling on cue. *Cell* **79**, 551–555 (1994).
4. Sherr, C.J. & Roberts, J.M. CDK inhibitors: Positive and negative regulators of G1-phase progression. *Genes Dev.* **13**, 1501–1512 (1999).
5. LaBaer, J. *et al.* New functional activities for the p21 family of CDK inhibitors. *Genes Dev.* **11**, 847–862 (1997).
6. Cheng, M. *et al.* The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J.* **18**, 1571–1583 (1999).
7. Koff, A., Ohtsuki, M., Polyak, K., Roberts, J.M. & Massague, J. Negative regulation of G1 in mammalian cells: Inhibition of cyclin E-dependent kinase by TGF-β. *Science* **260**, 536–539 (1993).
8. Polyak, K. *et al.* p27^{Kip1}, a cyclin-Cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. *Genes Dev.* **8**, 9–22 (1994).
9. Slingerland, J.M. *et al.* A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor β-arrested epithelial cells. *Mol. Cell. Biol.* **14**, 3683–3694 (1994).
10. Hannon, G.J. & Beach, D. p15^{INK4B} is a potential effector of TGF-β-induced cell cycle arrest. *Nature* **371**, 257–261 (1994).
11. Reynisdottir, I., Polyak, K., Iavarone, A. & Massague, J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. *Genes Dev.* **9**, 1831–1845 (1995).
12. Sandhu, C. *et al.* Transforming growth factor β stabilizes p15^{INK4B} protein, increases p15^{INK4B} -cdk4 complexes and inhibits cyclin D1/cdk4 association in human mammary epithelial cells. *Mol. Cell Biol.* **17**, 2458–2467 (1997).
13. Florenes, V.A. *et al.* Interleukin-6 dependent induction of the cyclin dependent kinase inhibitor p21WAF1/CIP1 is lost during progression of human malignant melanoma. *Oncogene* **18**, 1023–1032 (1999).
14. Bouchard, C. *et al.* Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. *EMBO J.* **18**, 5321–5333 (1999).
15. Cheng, M., Sexl, V., Sherr, C.J. & Roussel, M.F. Assembly of cyclin D-dependent kinase and titration of p27^{Kip1} regulated by mitogen-activated protein kinase kinase (MEK1). *Proc. Natl. Acad. Sci. USA* **95**, 1091–1096 (1998).
16. Perez-Roger, I., Kim, S.H., Griffiths, B., Sweing, A. & Land, H. Cyclins D1 and D2 mediate Myc-induced proliferation via sequestration of p27^{Kip1} and p21^{Cip1}. *EMBO J.* **18**, 5310–5320 (1999).
17. Warner, B.J., Blain, S.W., Seoane, J. & Massague, J. Myc downregulation by transforming growth factor β required for activation of the p15^{INK4B} G1 arrest pathway. *Mol. Cell Biol.* **19**, 5913–5922 (1999).
18. Vlach, J., Hennecke, S., Alevizopoulos, K., Conti, D. & Amati, B. Growth arrest by the cyclin-dependent kinase inhibitor p27^{Kip1} is abrogated by c-Myc. *EMBO J.* **15**, 6595–6604 (1996).
19. Slingerland, J. & Pagano, M. Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J. Cell Physiol.* **183**, 10–17 (2000).
20. Singh, S.P. *et al.* Loss or altered subcellular localization of p27 in Barrett's associated adenocarcinoma. *Cancer Res.* **58**, 1730–1735 (1998).
21. Bos, J.L. Ras oncogenes in human cancer: A review. *Cancer Res.* **49**, 4682–4689 (1989).
22. Di Cristofano, A. & Pandolfi, P.P. The multiple roles of PTEN in tumor suppression. *Cell* **100**, 387–390 (2000).
23. Diehl, J.A., Cheng, M., Roussel, M.F. & Sherr, C.J. Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* **12**, 3499–3511 (1998).
24. Sun, H. *et al.* PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,trisphosphate and Akt/protein kinase B signaling pathway. *Proc. Natl. Acad. Sci. USA* **96**, 6169–6204 (1999).
25. Medema, R.H., Kops, G.J., Bos, J.L. & Burgering, B.M. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27^{Kip1}. *Nature* **404**, 782–787 (2000).
26. Alessi, D.R. *et al.* Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15**, 6541–6551 (1996).
27. Wang, Q. *et al.* Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol. Cell Biol.* **19**, 4008–4018 (1999).
28. Muslin, A.J. & Xing, H. 14-3-3 proteins: Regulation of subcellular localization by molecular interference. *Cell Signal* **12**, 703–709 (2000).
29. Obata, T. *et al.* Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J. Biol Chem.* **275**, 36108–36115 (2000).
30. Reynisdottir, I. & Massague, J. The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2. *Genes Dev.* **11**, 492–503 (1997).
31. Zeng, Y., Hirano, K., Hirano, M., Nishimura, J. & Kanaide, H. Minimal requirements for the nuclear localization of p27^{Kip1}, a cyclin-dependent kinase inhibitor. *Biochem. Biophys. Res. Commun.* **274**, 37–42 (2000).
32. Jans, D.A. & Hubner, S. Regulation of protein transport to the nucleus: Central role of phosphorylation. *Physiol Rev.* **76**, 651–685 (1996).
33. Catzavelos, C. *et al.* Decreased levels of the cell-cycle inhibitor p27^{Kip1} protein: Prognostic implications in primary breast cancer. *Nature Med.* **3**, 227–230 (1997).
34. Zhou, B.P. *et al.* Cytoplasmic localization of p21$^{Cip1/WAF1}$ by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nature Cell Biol.* **3**, 245–252 (2001).
35. Rossig, L. *et al.* Akt-Dependent Phosphorylation of p21^{Cip1} regulates PCNA binding and proliferation of endothelial cells. *Mol. Cell Biol.* **21**, 5644–5657 (2001).
36. Li, Y., Dowbenko, D. & Lasky, L.A. AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21$^{Cip/WAF1}$ and promotes cell survival. *J. Biol. Chem.* **277**, 11352–11361 (2002).
37. Tsihlias, J., Kapusta, L. & Slingerland, J. The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer. *Annu. Rev. Med.* **50**, 401–423 (1999).
38. Yang, H.-Y., Zhou, B.P., Hung, M.-C. & Lee, M.-H. Oncogenic signals of HER-2/neu in regulating the stability of the cyclin-dependent kinase inhibitor p27. *J. Biol. Chem.* **275**, 24735–24739 (2000).
39. Donovan, J.C., Milic, A. & Slingerland, J.M. Constitutive MEK/MAPK activation leads to p27^{Kip1} deregulation and antiestrogen resistance in human breast cancer cells. *J. Biol. Chem.* **276**, 40888–40895 (2001).
40. Coffer, P.J., Jin, J. & Woodgett, J.R. Protein kinase B (c-Akt): A multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem. J.* **335**, 1–13 (1998).
41. Stampfer, M. Isolation and growth of human mammary epithelial cells. *J. Tissue Cult. Methods* **9**, 107–115 (1985).
42. Soule, H.D., Vazguez, J., Long, A., Albert, S. & Brennan, M. A human cell line from a pleural effusion derived from a breast carcinoma. *J. Natl. Cancer Inst.* **51**, 1409–1416 (1973).
43. Herlyn,M. Human melanoma: Development and progression. *Cancer Metastasis Rev.* **9**, 101–112 (1990).
44. Adam, S.A., Sterne-Marr, R. & Gerace, L. Nuclear protein import using digitonin-permeabilized cells. *Methods Enzymol.* 97–110 (1992).
45. Franke, T.F. *et al.* The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**, 727–736 (1995).

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Altered p27^{Kip1} Phosphorylation, Localization, and Function in Human Epithelial Cells Resistant to Transforming Growth Factor β-Mediated G_1 Arrest

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p27^{Kip1} is an important effector of G_1 arrest by transforming growth factor β (TGF-β). Investigations in a human mammary epithelial cell (HMEC) model, including cells that are sensitive (184^S) and resistant (184A1L5^R) to G_1 arrest by TGF-β, revealed aberrant p27 regulation in the resistant cells. Cyclin E1–cyclin-dependent kinase 2 (cdk2) and cyclin A-cdk2 activities were increased, and p27-associated kinase activity was detected in 184A1L5^R cells. p27 from 184A1L5^R cells was localized to both nucleus and cytoplasm, showed an altered profile of phosphoisoforms, and had a reduced ability to bind and inhibit cyclin E1-cdk2 in vitro when compared to p27 from the sensitive 184^S cells. In proliferating 184A1L5^R cells, more p27 was associated with cyclin D1-cdk4 complexes than in 184^S. While TGF-β inhibited the formation of cyclin D1-cdk4-p27 complexes in 184^S cells, it did not inhibit the assembly of cyclin D1-cdk4-p27 complexes in the resistant 184A1L5^R cells. p27 phosphorylation changed during cell cycle progression, with cyclin E1-bound p27 in G_0 showing a different phosphorylation pattern from that of cyclin D1-bound p27 in mid-G_1. These data suggest a model in which TGF-β modulates p27 phosphorylation from its cyclin D1-bound assembly phosphoform to an alternate form that binds tightly to inhibit cyclin E1-cdk2. Altered phosphorylation of p27 in the resistant 184A1L5^R cells may favor the binding of p27 to cyclin D1-cdk4 and prevent its accumulation in cyclin E1-cdk2 in response to TGF-β.

Transforming growth factor-beta (TGF-β) arrests or delays G_1 progression in normal epithelial cells (22). In contrast, most cancer-derived cell lines show some resistance to G_1 arrest by TGF-β, and in advanced cancers, TGF-β may stimulate proliferation and metastatic progression (11). In this study, we investigated cell cycle effects of TGF-β and how p27, a key effector of arrest by TGF-β, is regulated using finite-life-span, normal human mammary epithelial cell (HMEC) strain 184, which is sensitive to TGF-β (16), and an immortal derivative of 184, 184A1L5, which has undergone conversion to TGF-β resistance (46). For clarity, superscripts S (184^S) and R (184A1L5^R) indicate sensitivity and resistance to growth arrest by TGF-β.

Cell cycle progression through G_1 phase is regulated by the sequential activation of D-type cyclin and then E-type cyclin-dependent kinases (cdk's) (37). Cyclin A-cdk2 is required for S-phase progression. cdk2 and cdk4 are both activated by cdk-activating kinase (CAK) (42), and cdk2 activation also requires dephosphorylation of inhibitory sites by Cdc25A (9). Both of these cdk-activating mechanisms are inhibited by TGF-β (17, 20, 41). G_1 cdk's are also regulated by two families of cdk inhibitors (38, 39): the kinase inhibitor proteins (KIP) p21, p27, and p57 and the inhibitors of cdk4 (INK4) p15, p16, p18, and p19. Both p21 and p27 contribute to TGF-β arrest (reviewed in reference 7) and both *p15* gene transcription (13, 55), and p15 protein stability (35) are increased by TGF-β.

p27 was first discovered as a heat-stable inhibitor of cyclin E-cdk2 in TGF-β-arrested cells (20, 33, 41). While p27 mRNA is constant across the cell cycle, p27 protein levels are regulated by translational controls (15, 28) and by ubiquitin-dependent proteolysis (32). p27 is maximal in G_0, and phosphorylation at T187 in late G_1 targets its proteasomal degradation (29, 36, 54). The cyclin E-cdk2-inhibitory activity of p27 is maximal in G_0 and falls as cells move through G_1 into S phase (14, 33, 41).

p27 plays two roles during G_1-to-S phase progression. In addition to inhibition of cyclin E-cdk2, p27 also acts early in G_1 to facilitate assembly, activation, and nuclear localization of cyclin D-cdk complexes (5, 21). Polyak et al. showed that p27's cyclin E-cdk2 inhibitory activity could be titrated out of TGF-β-arrested cell lysates by addition of recombinant cyclin D2-cdk4 (33). This seminal observation led to the hypothesis that the abundance of cyclin D1 may regulate p27 function, with p27 shifting out of cyclin E-cdk2 complexes into cyclin D-cdk4 complexes during G_1 progression. It has been proposed that a key noncatalytic function of cyclin D-cdk's is to titrate p21 and p27 away from cyclin E-cdk2, allowing the activation of the latter in late G_1 (5, 33).

A number of observations suggest that p27 may not be passively regulated by the abundance of cyclin D1 alone. In most normal and many transformed cell types, cyclin D1-cdk4 activation precedes that of cyclin E-cdk2 by several hours and is not immediately or simultaneously accompanied by cyclin E1-cdk2 activation (2, 37). Moreover, despite its abundance in G_0,

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p27 is unable to assemble exogenously overexpressed cyclin D1 into cdk4 complexes in serum-starved fibroblasts (26). However, overexpression of cyclin D1 together with constitutively activated MEK leads to sequestration of p27 away from cyclin E and into cyclin D1-cdk4-p27 complexes (6). These observations, together with data presented herein, suggest that p27 may be actively regulated by phosphorylation to function either as an inhibitor of cyclin E1-cdk2 or as an assembly factor for cyclin D1-cdk's. The shift of p27 out of cyclin E1-cdk2 and into cyclin D1-cdk complexes may be regulated by mitogenic kinases acting early in G_1 and not merely passively regulated by the increasing abundance of cyclin D1 protein.

G_1 arrest by TGF-β is brought about by a series of complementary and redundant mechanisms (7, 25). In epithelial cells, inhibition of G_1 cyclin-cdk's by TGF-β involves the coordinate actions of p15 and p27 (34, 35). p15 induced by TGF-β was initially thought to dissociate p27 and cyclin D1 from cdk4 and promote the binding and inhibition of cyclin E-cdk2 by p27. Recent data suggest that, where p15 and p27 are both functional, they cooperate. However, cells lacking either p15 or p27 can still undergo TGF-β-mediated arrest (12, 17, 30). p15 is not required for binding and inhibition of cyclin E-cdk2 by p27, since cells bearing p15 deletions can still respond to TGF-β with accumulation of p27 in cyclin E-cdk2 and kinase inhibition (12, 17).

In HMECs, TGF-β mediates an increase in p15 stability and binding to cdk4, dissociation of p27 and cyclin D1 from cdk4, and binding and inhibition of cyclin E1-cdk2 by p27 in the sensitive 184^S cells but not in resistant $184A1L5^R$ cells (35). We showed that, while p15 from both cell types could dissociate in vitro cyclin D1-cdk4-p27 complexes isolated from 184^S cells, cyclin D1-cdk4-p27 complexes from the $184A1L5^R$ cells were resistant to dissociation by p15, suggesting an alteration in their conformation. The present investigation suggests that defective p27 regulation, with constitutive activation of p27 assembly function for cyclin D-cdk complexes, may underlie the TGF-β resistance in $184A1L5^R$. Our data suggest a model in which TGF-β regulates the affinity of p27 binding to its target cdk's by modulating p27 phosphorylation, shifting it from an assembly factor for D-type cyclin-cdk's to a form that binds and effectively inhibits cyclin E-cdk2.

MATERIALS AND METHODS

Culture of HMECs. The derivation of normal finite-life-span HMECs from specimen 184 has been described (44). The immortal cell line 184A1 was derived following exposure of 184 HMECs to benzo(a)pyrene (45). Early-passage $184A1^S$ is sensitive to growth inhibition by TGF-β. With progressive passage, cells undergo a gradual conversion process with outgrowth of a TGF-β-resistant population (46). Fully TGF-β-resistant cells can be readily isolated from late-passage 184A1 cells to give stable, growth-resistant sublines, such as $184A1L5^R$. The resistant subpopulation in $184A1^S$ cannot arise as a result of a single genetic event, since all of four separate single-cell clones of $184A1^S$ that were examined exhibited, when expanded, the presence of a TGF-β-resistant subpopulation. Thus, the switch from sensitive to resistant occurs at a population frequency that defies explanation on the basis of a single mutation. Hence, mutation of the cdk's, cyclins, or cdk inhibitors cannot underlie the conversion from TGF-β growth inhibited to growth resistant. Both 184^S and $184A1L5^R$ express normal TGF-β receptors and produce an extracellular matrix in response to TGF-β (16, 48).

HMECs were grown in MCDB 170 medium (Clonetics Corp., San Diego, Calif.). HMECs were G_0 arrested by blocking epidermal growth factor (EGF) receptor signal transduction as described earlier (47). Asynchronously growing HMECs were treated with 2.5 ng of TGF-β1 (Genentech Inc., San Francisco, Calif.)/ml for 48 h.

Production of cyclin E1-cdk2 by baculovirus infection of Sf-9 cells. Sf-9 cells and TNM-FH media were obtained from Invitrogen. Adherent Sf-9 cells were coinfected with baculoviruses expressing human *cyclin E1* or human *cdk2* genes, and cyclin E1 and cdk2 were prepared as described earlier (24). Sf-9 cell lysates containing cyclin E1 and cdk2 were used directly in p27 inhibitor assays and p27 binding assays.

Flow cytometry analysis. Cells were pulse labeled with 10 μM bromodeoxyuridine and processed for flow cytometry as described (35).

Antibodies. The anti-EGF receptor monoclonal antibody (MAb) 225 was provided by Steve Wiley (University of Utah Medical Center, Salt Lake City, Utah). Antibodies to cyclin A, cdk2, cdk4, cdk6, and p27 (C-19) were obtained from Santa Cruz Biotechnology; to cyclin D1 (DCS-6) and p27 (DCS-72) from Neomarkers; to PSTAIRE from S. Reed (The Scripps Research Institute, La Jolla, Calif.); and to cyclin E1 (MAbs E12 and E172) from E. Harlow (Massachusetts General Hospital, Boston, Mass.). These cyclin E1 antibodies are specific for cyclin E1 (24). Cyclin A MAb E67 was provided by J. Gannon and T. Hunt (Imperial Cancer Research Fund, London, England). p27 rabbit polyclonal serum (pAb5588) was provided by H. Toyoshima and T. Hunter (Salk Institute, La Jolla, Calif.). p27 and RCC1 antibodies were purchased from Transduction Laboratories.

Immunoblotting. Cell lysis and immunoblotting were performed as described earlier (35). Equal protein loading was verified by blotting for β-actin. To assay cyclin E1-cdk-p27 complexes, cyclin E1 or p27 was immunoprecipitated from 200 μg of protein lysate. Immunoprecipitates were resolved, transferred, and blotted with cyclin E1, cdk2, and p27 antibodies. Proteins were detected by enhanced chemiluminescence (ECL). Antibody-alone controls were run along side all immunoprecipitates.

To compare the relative amounts of cyclin-bound p27, lysates from actively proliferating or G_0-arrested cells were serially immunoprecipitated with cyclin D1 antibody three times. Cyclin E1 was then serially immunoprecipitated three times, followed by a final precipitation with anti-p27 antibody (C19). For G_0 cells, cdk4 and cdk6 were immunoprecipitated prior to p27 precipitation. Precipitates were resolved and immunoblotted for associated proteins. The relative amounts of cyclin D1 and cyclin D1-bound p27 were measured by densitometric analysis of two different exposures on Western blots from each of two different biologic experiments. The relative amounts of cyclin D1 and of p27 bound to cyclin D1 were graphed as a percentage of the total in TGF-β-resistant $184A1L5^R$. For G_0 lysates, cyclin D1-, E1-, cdk4- and cdk6-bound p27 and remaining p27 were quantitated from different ECL exposures from two different experiments.

cdk assays. Cyclin E1, cyclin A, or p27 complexes were immunoprecipitated and reacted with [γ-^{32}P]ATP and histone H1 as described earlier (10, 41). Radioactivity incorporated in histone substrate was quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software. Radioactivity incorporated in control nonspecific mouse or rabbit polyclonal immunoglobulin G (IgG) immunoprecipitates was subtracted from test kinase values prior to quantitation of differences between activities in different cell types or different conditions.

Metabolic labeling. Cells were labeled metabolically for 1 h with 500 μCi of [^{35}S]methionine as described earlier (35). Lysates were precleared and incorporation of radioactivity was assayed by quantitation of trichloroacetic acid-insoluble counts. Volumes representing 10^8 cpm of trichloroacetic acid-insoluble radioactivity were precipitated with either cdk4 or cyclin D1 antibodies or nonimmune serum. Immune complexes were resolved, gels were dried, and proteins were visualized by autoradiography.

p27 immunocytochemistry. HMECs were grown to 60% confluence on glass slides and were then depleted of EGF for 48 h. Cells were fixed and p27 immunoreactivity was detected using p27 MAb from Transduction Laboratories, as described earlier (4). Isotype-specific polyclonal mouse IgG was used in place of primary antibody for negative controls. The pattern of p27 immunostaining was confirmed using the polyclonal C-19 p27 antibody (Santa Cruz Biotechnology).

Nuclear cytoplasmic fractionation. HMECs arrested by EGF depletion for 48 h were harvested and resuspended in ice-cold buffer (20 mM HEPES, pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM $Mg[OAc]_2$, 1 mM EGTA, and 2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin/ml, and 1 μg of aprotinin/ml). The cells were permeabilized at 4°C for 5 min by gradual addition of 2 to 3 μl of 0.1% digitonin until 95 to 98% permeabilization was achieved (detected by trypan blue staining). Nuclei were isolated by centrifugation, and the supernatant containing the cytosolic fraction was collected. The nuclei were washed, and ice-cold 0.1% NP-40 lysis buffer (see above for immu-

noblotting) was added to both fractions. To verify the adequacy of fractionation, immunoblots were probed for the nuclear protein RCC1.

Transfection of HMECs with fluorescent-tagged p27 and p27 localization. A vector encoding wild-type human p27 cDNA fused to the yellow-green variant of the *Aequorea victoria* green fluorescent protein (YFPp27wt) was prepared in the pEYFP-C1 vector from Clontech Laboratories. HMECs were grown on glass slides to 60% confluence, depleted of EGF, and then transfected with the YFPp27wt vector using Lipofectamine PLUS Reagent (Life Technologies) according to the manufacturer's instructions. Fluorescent-tagged p27 was observed with a Zeiss Axiovert S100TV microscope with a C-Aprochromat 40× objective. Photographs were taken at 400× magnification, using an Empix digital camera and "CoolSnap" software.

Assays of p27 inhibitor function and binding to cyclin E1-cdk2. 184^S or $184A1L5^R$ cells were arrested in G_0 by EGF depletion for 42 h following 48 h of contact inhibition. Fifty- to 300-μg cell lysates were precipitated with pAb5588 anti-p27 serum or control polyclonal rabbit IgG and protein A-Sepharose beads. For testing unboiled p27 inhibitory and binding activity, p27 immunoprecipitates were washed three times with 0.1% NP-40 lysis buffer and finally with reaction buffer (20 mM Tris, pH 7.5, 7.5 mM $MgCl_2$, and 1 mM DTT). The immunoprecipitated p27 was incubated with a molar excess of recombinant cyclin E1-cdk2 at 30°C for 30 min. Complexes were then assayed either for H1 kinase activity or immunoblotted to detect associated proteins. The kinase activity of "uninhibited" recombinant cyclin E1-cdk2 was assayed following admixture with nonspecific IgG antibody control precipitates.

For testing heat-stable p27 inhibitor and cyclin E1-cdk2 binding activity, p27 immunoprecipitates were washed and then boiled for 5 min in 200 μl of reaction buffer, placed on ice, and then cleared by centrifugation. The p27 in the supernatant was recovered, recombinant cyclin E1-cdk2 and DTT (1 mM final concentration) were added, and the mixture was incubated at 30°C for 30 min, followed by immunoprecipitation with either anti-cyclin E1 (MAb E172) or control polyclonal mouse IgG (Sigma). Complexes were then either assayed for H1 kinase activity or immunoblotted to detect cyclin E1-bound cdk2 and p27.

2DIEF and phosphatase treatment. Cells were lysed in ice-cold 0.1% Tween 20 lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 2.5 mM EGTA, pH 8.0, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na_2VO_4, 0.5 mM DTT, and 0.02 mg each of aprotinin, leupeptin, and pepstatin per ml). For two-dimensional isoelectric focusing (2DIEF), p27 immunoprecipitates were denatured in 8 M urea, loaded onto immobilized linear pH gradient (pH 3 to 10) or nonlinear (pH 3 to 10) IEF strips, and focused for 50,000 V·h, using the IPGphor apparatus from Amersham Pharmacia. The IEF strip was equilibrated in 50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, and 2% sodium dodecyl sulfate (SDS) for 30 min before loading for SDS-polyacrylamide gel electrophoresis (PAGE). Gels were transferred to a polyvinyl difluoride membrane, and p27 isoforms were detected by immunoblotting using p27 antibody from Transduction Laboratories. For 2DIEF of cyclin D1-or cyclin E1-bound p27, 2 to 5 mg of protein lysate was precipitated with the appropriate antibody. For phosphatase treatment, p27 immunoprecipitates were washed twice with phosphatase buffer (50 mM Tris, pH 8.0, and 10% glycerol) and were then incubated at 37°C for 3 h with 10 U per 10-μl reaction of calf intestinal alkaline phosphatase (Boehringer Mannheim). For orthophosphate labeling, cells were transferred to phosphate-free medium for 1 h prior to labeling with 1 mCi of [^{32}P]orthophosphate per p100 dish for 3 h. p27 was immunoprecipitated, and then complexes were resolved by 2DIEF. Immunoblotted p27 was detected by ECL and then exposed to film for autoradiography.

RESULTS

p27-cyclin-cdk2 complexes in TGF-β-resistant cells have increased kinase activity. To investigate p27 effects on cdk2 complexes, lysates were prepared from asynchronous 184^S (passage 12) and $184A1L5^R$ populations with similar cell cycle distributions. 184^S cells had 68% G_1-, 25% S-, and 17% G_2/M-phase cells, while the $184A1L5^R$ cells had 65% G_1-, 28% S-, and 17% G_2/M-phase cells. The doubling times of the two cell types were similar at approximately 26 h. Levels of cyclin and cdk expression were quantitated by densitometry of different ECL exposures to ensure that the band densities were in the linear range of the film. Equal protein loading was verified by reprobing for β-actin. While the amounts of cyclin E1 and cyclin E1-bound cdk2 were similar, cyclin E1-cdk2 activity was higher in the resistant cells than in the sensitive cells (Fig. 1A). Similarly, cyclin A-dependent kinase activity was also elevated in $184A1L5^R$ (Fig. 1A), despite similar levels of cyclin A and cyclin A-bound cdk2.

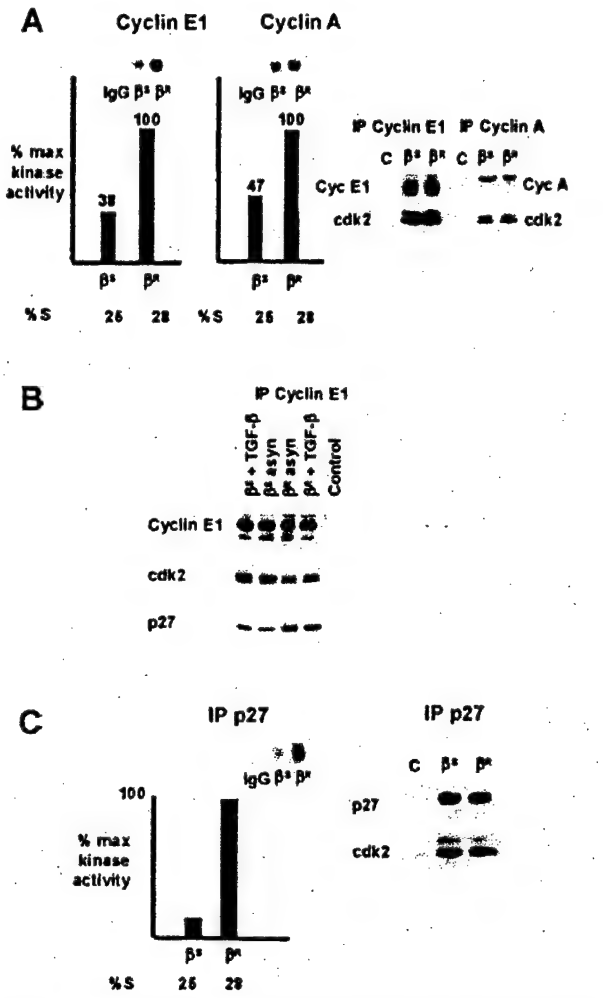

FIG. 1. Cyclin-cdk2-p27 complexes and kinase activities. Cell lysates were prepared from asynchronously growing 184^S (β^S) and $184A1L5^R$ (β^R) cells that had similar cell cycle distributions (% S phase indicated). (A) Cyclin E1- and cyclin A-associated kinase activities. Cyclin E1 and cyclin A immunoprecipitates (IP) were assayed for associated kinase activities (left) or resolved by SDS-PAGE, transferred, and immunoblotted for cyclin and associated cdk2 (right) as described in Materials and Methods. For quantitation of the kinase reactions, radioactivity in nonspecific immune control (IgG) was subtracted and kinase activities were plotted as a percentage of the maximum (% max). Radioactivity in histone H1 bands is shown in the graph inset. (B) Cyclin E1 immunoprecipitated from asynchronously growing or TGF-β-treated 184^S (β^S) or $184A1L5^R$ (β^R). Complexes were resolved and proteins were detected by immunoblotting as indicated. Antibody-only control is shown on the right. (C) p27 immunoprecipitates in $184A1L5^R$ cells contain active kinase. p27 immunoprecipitates from asynchronous HMECs with similar cell cycle distributions were assayed for cdk2 activity using histone H1 as substrate or resolved and immunoblotted for p27 and associated cdk2. Radioactivity in the nonspecific immune control (IgG) was subtracted prior to quantitation as for panel A above.

Cyclin E1-bound cdk2 in proliferating 184A1L5^R cells showed an increase in the proportion of CAK-activated faster-mobility cdk2 isoform from that in 184^S cells (Fig. 1A and B). Despite the higher cyclin E1-cdk2 activity, there was no loss of cyclin E1-bound p27 in proliferating 184A1L5^R cells. Indeed, cyclin E1-bound p27 was somewhat increased in asynchronously growing resistant cells. These data raised the possibility that p27 from the 184A1L5^R might bind cyclin E1-cdk2 with an altered conformation, possibly permitting cdk2 activity. To test this, equal amounts of p27 were precipitated from the two cell lines and were assayed for associated histone H1 kinase activity (Fig. 1C, left), and parallel p27 immunoprecipitations were immunoblotted to detect p27 and associated cdk2 (Fig. 1C, right). Asynchronously growing sensitive and resistant cells expressed p27 protein at similar levels. p27 immunoprecipitated from proliferating 184A1L5^R showed significant histone H1 kinase activity, while that from 184^S cells was only minimally above the nonspecific activity detected in nonimmune control precipitates. The amounts of cdk2 bound to p27 in asynchronous 184^S and 184A1L5^R cells were similar.

TGF-β inhibits cyclin D1-cdk4-p27 assembly in 184^S but not in 184A1L5^R cells. The ability of newly synthesized cyclin D1 and cdk4 to assemble into cyclin D1-cdk4-p27 complexes was assayed by metabolic labeling in 184^S and 184A1L5^R cells. The HMECs were pulse labeled with [^{35}S]methionine after 48 h of EGF depletion (G_0 arrest) or at 12 h after release from G_0 by readdition of EGF without (mid-G_1) or with addition of TGF-β. Metabolically labeled cyclin D1 and cdk4 complexes are shown in Fig. 2. In G_0, newly synthesized cyclin D1 precipitates did not bind cdk4, and cyclin D1 and p27 were barely detectable in cdk4 precipitates. In mid-G_1, at a time when cyclin D1-cdk4 is active (35), cdk4 immunoprecipitates contained both cyclin D1 and p27. Although p27 is abundant in EGF-depleted, G_0-arrested HMECs (see Fig. 5), it does not appear to facilitate the assembly of newly synthesized cyclin D1 with cdk4 (Fig. 2). Thus, the cyclin D1-cdk assembly function of p27 is lacking in G_0, and p27 may require posttranslational modification to function as an assembly factor as cells move from G_0 to mid-G_1. When TGF-β was added to 184^S cells at the same time that cells were stimulated to reenter cell cycle by readdition of EGF, the association of p27 and cyclin D1 with cdk4 was inhibited. While TGF-β caused a modest reduction in synthesis of cdk4 in 184^S cells, it caused a more notable reduction in the relative amounts of cdk4-associated cyclin D1 and p27. In contrast, TGF-β did not prevent the assembly of p27-cyclin D1-cdk4 in resistant 184A1L5^R cells.

p27 from 184A1L5^R cells is more cyclin D1 bound than p27 from 184^S cells. The steady-state levels of cyclin D1-bound p27 in asynchronous cells were compared by immunoprecipitation-Western blotting in the two lines. In addition to the differences in p27's functional association with cyclin E1-cdk2 complexes between the two lines (Fig. 1), proliferating 184A1L5^R cells also showed an almost-twofold increase in steady-state binding of p27 to cyclin D1 from the level in 184^S cells (Fig. 3A and B). Cyclin D1 immunoprecipitated from the resistant cells consistently showed, on repeat assays, a greater amount of associated p27 than did cyclin D1 from asynchronously proliferating 184^S cells. p27 binding to cyclin D1 was corrected for the minor difference in total cyclin D1 immunoprecipitated from the two cell types and was graphed as a percentage of that observed in

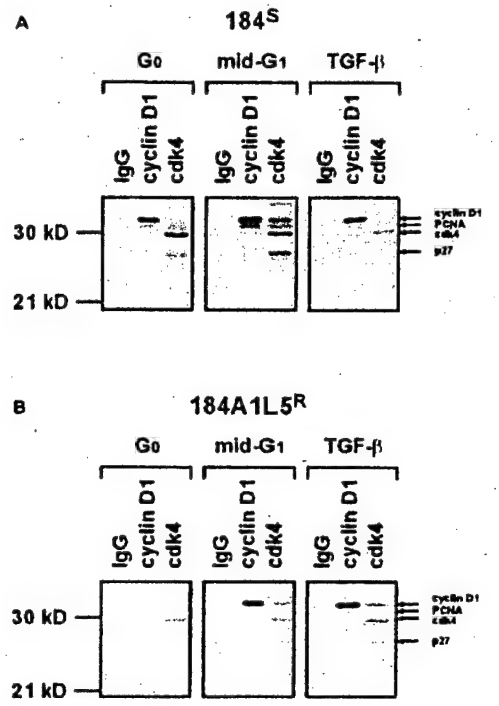

FIG. 2. p27-cyclin D1-cdk4 assembly detected by metabolic labeling. 184^S (A) and 184A1L5^R (B) were grown to 60% confluence and were then arrested by EGF deprivation (G_0). Cells were then transferred to complete medium and cultured for 12 h without (mid-G_1) or with addition of TGF-β. At the times indicated, cells were pulse labeled with [^{35}S]methionine and were cyclin D1 or cdk4 immunoprecipitated. Nonimmune controls (IgG) were run alongside cyclin D1-cdk4 precipitates. Cyclin D1, PCNA, cdk4, and p27 are indicated with arrows on the right. Molecular weight markers in kilodaltons (kD) are indicated on the left.

184A1L5^R in Fig. 3B. Moreover, the cyclin D1-bound p27 showed a slower mobility on SDS-PAGE than did cyclin E1-bound p27 (Fig. 3C). Cyclin E1-bound p27 from G_0 cells had a mobility similar to that in asynchronous cells (not shown). The possibility that these different mobility forms of p27 may reflect altered p27 phosphorylation in cyclin D1 from that in cyclin E1 complexes was pursued further by 2DIEF (see below).

p27 from 184A1L5^R cells has a reduced ability to bind and inhibit cyclin E1-cdk2. To test p27 inhibitory function directly, p27 was immunoprecipitated and its ability to bind and inhibit recombinant cyclin E1 and glutathione transferase (GST)-tagged cdk2 complexes (cyclin E1-cdk2$_{GST}$) was assayed. To ensure that differences in p27 function were not due to subtle differences in the cell cycle profiles between the two cell types, cells were G_0 arrested by contact inhibition and EGF depletion (88 to 90% of cells with 2 N DNA content and <2% of cells in S phase). The amounts of p27 to be compared from the two cell types were titrated by immunoblotting and were arbitrarily designated 0.5×, 1×, and 2×. p27 immunoprecipitates from G_0-arrested 184^S cells contained more associated endogenous cellular cyclin E1 and cdk2 than did p27 from quiescent 184A1L5^R cells (left side, Fig. 4A). On addition of recombinant cyclin E1-cdk2$_{GST}$, approximately twice as much cyclin

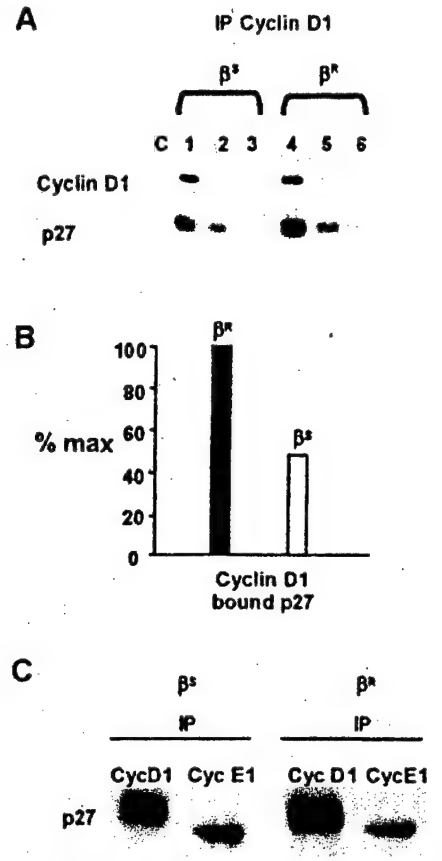

FIG. 3. p27 binding to cyclin D1 and cyclin E1. (A) Asynchronously growing 184^S (β^S) or $184A1L5^R$ (β^R) cell lysates containing 200 μg of protein were serially immunodepleted of cyclin D1. Lanes 1 to 3 and 4 to 6 represent serial cyclin D1 immunoprecipitation (IP) from β^S and β^R cells, respectively. Immune complexes were resolved by SDS-PAGE and transferred, and blots were probed for cyclin D1 and p27. Lane C, control. (B) Cyclin D1 and p27 levels were quantitated by densitometry from different exposures of the blots in panel A, and the amounts of cyclin D1-bound p27 were corrected for the difference in total cyclin D1 between the two lines and graphed as a percentage of the maximum (% max) of that seen in the β^R cells. (C) The mobilities of cyclin D1 (CycD1)- and cyclin E1 (CycE1)-bound p27 from asynchronous β^S and β^R cells are shown. Fifty micrograms of lysate was used for the cyclin D1 immunprecipitation, and 250 μg of lysate was used for cyclin E1 precipitation.

E1-cdk2$_{GST}$ bound to p27 from 184^S cells than to p27 from $184A1L5^R$ cells (right side, Fig. 4A).

The cyclin E1-cdk2 inhibitory function of p27 was assayed using the same G_0-arrested 184^S or $184A1L5^R$ lysates as for Fig. 4A. Recombinant cyclin E1-cdk2$_{GST}$ was incubated with the indicated amounts of immunoprecipitated p27 from each cell type. Uninhibited cyclin E1-cdk2$_{GST}$ activity was quantitated and compared with the activity remaining after admixture of cellular p27. The cyclin E1-cdk2-inhibitory activity of p27 from 184^S cells was approximately twice that of p27 from $184A1L5^R$ cells (Fig. 4B). The data graphed are the mean of three kinase inhibition assays.

To test if this difference in inhibitory activities reflected different amounts of free, non-cyclin-bound p27, cyclins and cdk's were immunodepleted from G_0 lysates and the amount of residual p27 was assayed by immunoblotting. Serial immunodepletion of cyclin D1, cyclin E1, and then of cdk4 and -6 followed by p27 precipitation and p27 immunoblotting showed a significant excess of p27 remaining in the final immunoprecipitation in both 184^S and $184A1L5^R$ G_0 lysates (80 and 67% of total, respectively, as assayed by densitometry, data not shown). The non-cyclin D1- and E1-bound p27 in 184^S cells was only 1.2-fold higher than that in $184A1L5^R$, and this difference in potentially unbound p27 could not fully account for the increased inhibitory activity in the 184^S lysates. The cyclin E immunodepletion confirmed that G_0 184^S cells had more cyclin E1-bound p27 than did $184A1L5^R$ cells. There was a modest amount of cyclin D1-bound p27 in the resistant cells, but cyclin D1-bound p27 was negligible in G_0 184^S cells. Thus, sequestration by cyclin D1 was not sufficient to account for the difference in inhibitory activities shown in Fig. 4B.

Reduced ability to bind and inhibit cyclin E1-cdk2 is a heat-stable property of p27 from $184A1L5^R$. As a second measure to rule out p27 sequestration by associated heat-labile cyclins or novel proteins, we made use of the heat-stable property of p27. p27 immunoprecipitated from 184^S or $184A1L5^R$ cells was boiled (100°C for 5 min). Under these conditions, p27 is essentially monomeric (14, 41). The recovered heat-stable p27 was incubated with cyclin E1-cdk2$_{GST}$. The binding of p27 to recombinant cyclin E1 was detected by immunoblotting after cyclin E1 immunoprecipitation. Less heat-stable p27 from $184A1L5^R$ cells bound to the recombinant cyclin E1-cdk2$_{GST}$ than did p27 from the 184^S cells (Fig. 4C). The difference in the kinase-inhibitory activities of heat-stable p27 from the two cell types was essentially the same as that observed for Fig. 4B. Approximately twice as much heat-stable p27 from the resistant cells was required to achieve the same inhibition of cyclin E1-cdk2$_{GST}$ as from the 184^S cells (not shown). Thus, the reduced ability of the heat-stable p27 from $184A1L5^R$ cells to bind and inhibit cyclin E1-cdk2 was not due to sequestration by cyclins nor likely due to the presence of a novel p27-associated protein but might rather reflect a heat-stable, posttranslational modification.

p27 shows altered intracellular localization in resistant cells. Some advanced cancer-derived cell lines show mislocalization of p27 in the cytoplasm (31). To test whether the loss of p27 function in $184A1L5^R$ cells was associated with altered cellular localization, the immunolocalization of p27 was assayed. p27 was predominantly nuclear in 184^S cells, while quiescent $184A1L5^R$ cells showed both nuclear and strong cytoplasmic p27 staining (Fig. 5A). Subcellular fractionation and immunoblotting confirmed a nuclear-to-cytoplasmic ratio of p27 in $184A1L5^R$ cells of approximately 1.5:1, while that in the 184^S cells was greater than 6:1 (representative blot, Fig. 5B). The nuclear protein RCC1 provided a control for fractionation (27). Similarly, TGF-β-arrested 184^S cells showed nuclear p27 localization, while TGF-β-treated $184A1L5^R$ cells showed both nuclear and cytoplasmic p27 staining (not shown). Moreover, when 184^S and $184A1L5^R$ cells were transiently transfected with a vector encoding YFPp27wt, the p27 fluorescence detected 24 h posttransfection was exclusively nuclear in 184^S cells but both cytoplasmic and nuclear in $184A1L5^R$ cells (Fig. 4C).

When nuclear and cytoplasmic fractions were compared from the two cell lines, equal amounts of p27 from both nu-

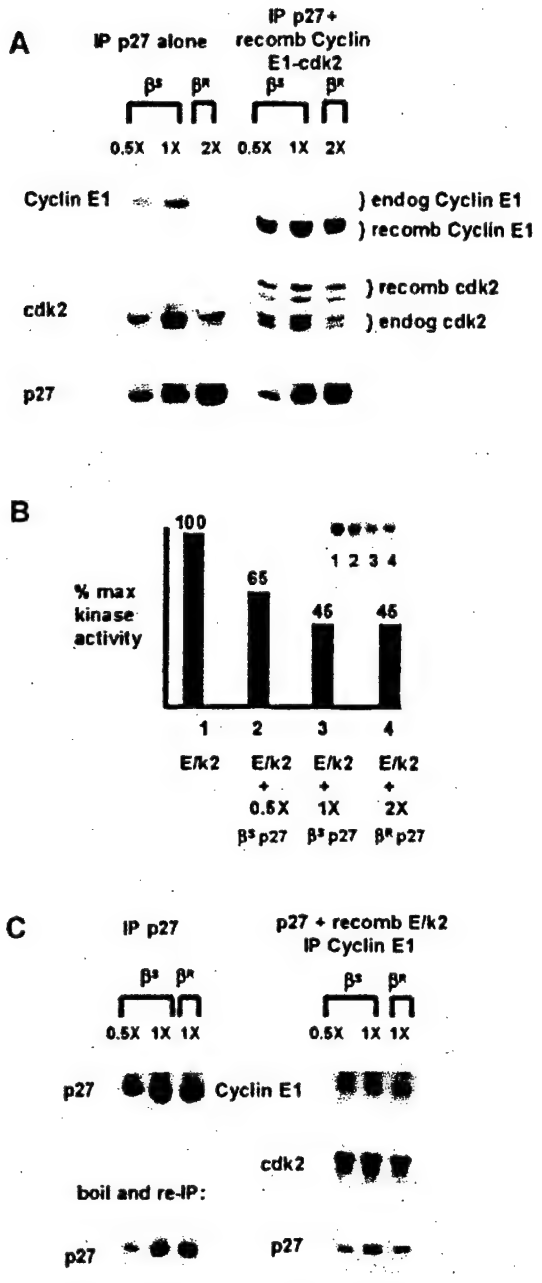

FIG. 4. In vitro assays of p27's ability to bind and inhibit cyclin E1-cdk2. (A) Titrated amounts of p27 were immunoprecipitated (IP) from G_0-arrested 184^S (β^S) or $184A1L5^R$ (β^R) cells, resolved by SDS-PAGE, and associated cellular cyclin E1 and cdk2 (endogenous) detected by immunoblotting (left panels). On the right, the indicated amounts of immunoprecipitated p27 from β^S and β^R cells were mixed with recombinant (recomb) cyclin E1 and GST-tagged cdk2 and were then resolved by SDS-PAGE and p27-bound endogenous (endog) and recombinant proteins detected by immunoblotting. (B) The inhibitory activity of p27 is reduced in $184A1L5^R$. Recombinant cyclin E1-cdk2 (recomb E/k2) was mixed with the indicated amounts of p27 immunoprecipitated from the same 184^S (β^S) or $184A1L5^R$ (β^R) cell lysates as for panel A above, and histone H1 kinase activity was assayed. Inhibition of E/k2 activity by the addition of p27 is shown. Radioactivity incorporated into histone H1 is shown in the autoradiograph (inset, upper right) and graphed as a percentage of the maximum (% max) uninhibited E/k2 activity. (C) The reduced affinity for E/k2 is a heat-stable property of p27 from $184A1L5^R$ cells. The indicated amounts of immunoprecipitated p27 from β^S and β^R cells were boiled (100°C for 5 min). The initial amount of p27 used is indicated in the immunoblot (top left), and p27 reprecipitated after boiling is shown (lower left). The free heat-stable p27, recovered after p27 complexes were boiled, was mixed with recombinant cyclin E1 and cdk2 proteins. Cyclin E1 was then immunoprecipitated, and the amount of cyclin E1-bound cellular p27 was detected by blotting. Results shown in panels A to C are a representative of at least three experiments.

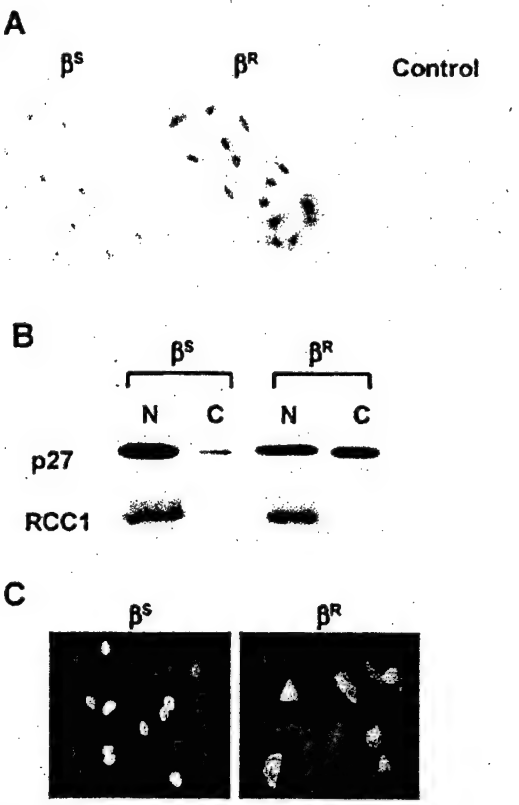

FIG. 5. p27 shows increased cytoplasmic localization in the $184A1L5^R$ cells. (A) Immunocytochemistry shows strong nuclear p27 staining in G_0-arrested 184^S (β^S, left). p27 is localized in both nucleus and cytoplasm in G_0-arrested $184A1L5^R$ cells (β^R, center). The negative control in $184A1L5^R$ cells is shown (right). (B) p27 content of nuclear (N) and cytoplasmic (C) fractions of quiescent β^S and β^R cells was assayed by Western blotting. (C) β^S and β^R cells were transfected with fluorescence-tagged p27 (YFPp27wt), and 24 h after transfection, p27 was detected by fluorescence microscopy. Cells in panels A and C were photographed at 400× magnification.

clear and cytoplasmic fractions from $184A1L5^R$ showed a decreased ability to bind recombinant cyclin E1-$cdk2_{GST}$, compared to nuclear p27 from the 184^S cells (not shown). Thus, the reduced cyclin E1-cdk2 binding affinity of total cellular p27 from $184A1L5^R$ cells could not be attributed solely to the presence of a dysfunctional p27 localized in the cytoplasm.

p27 phosphorylation is altered in TGF-β-resistant cells. 2DIEF showed that the differences in p27 function and localization between the 184^S and $184A1L5^R$ cells were associated with differences in p27 phosphorylation. In G_0 cells, at least seven different p27 isoforms could be reproducibly distin-

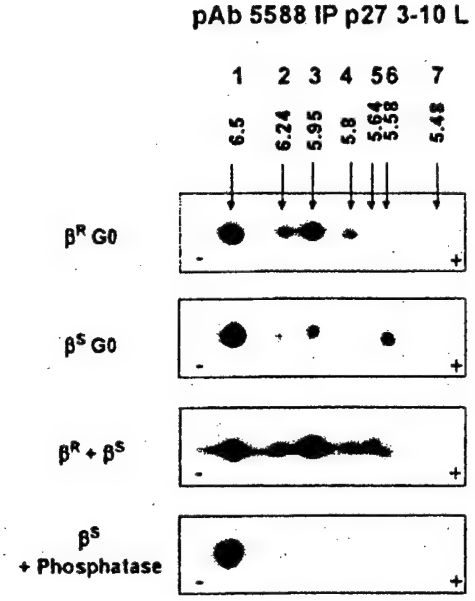

FIG. 6. p27 phosphorylation differs between 184^S and $184A1L5^R$ cells. p27 was immunoprecipitated (IP) using polyclonal p27 pAb5588 from G_0-arrested 184^S (β^S) or $184A1L5^R$ (β^R) cells. For 2DIEF, complexes were resolved using the IEF strips with a linear gradient of pH 3 to 10 (3-10 L) followed by SDS–12% PAGE. Gels were transferred and p27 isoforms were detected by immunoblotting with the Transduction Laboratories p27 antibody. The IEF points (pI) of the p27 isoforms (forms 1 to 7) are indicated with arrows. In the third panel, the p27 immunprecipitates from β^S and β^R cells were mixed prior to 2DIEF. The bottom panel shows that, after phosphatase treatment, most of the p27 migrated at the highest IEF point, 6.5.

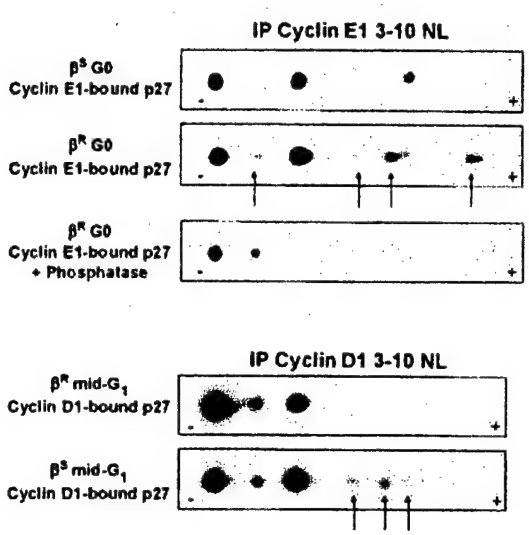

FIG. 7. The phosphorylation of cyclin E1-bound p27 differs from that of cyclin D1-bound p27. The 2DIEF profile of p27 in the 184^S (β^S) or $184A1L5^R$ (β^R) cells was assayed in cyclin E1 immune complexes from G_0 (contact inhibition for 48 h, followed by 48 h of EGF deprivation) (A) and in cyclin D1 immune complexes from mid-G_1 (B). Cyclin E1-bound p27 was treated with calf intestinal alkaline phosphatase prior to 2DIEF for the lower part of panel A. Complexes were resolved on pH 3 to 10 nonlinear gradient (3-10 NL) IEF strips prior to resolution on SDS–12% PAGE and immunoblotting for p27. The arrows (A and B) indicate cyclin-associated p27 isoforms in $184A1L5^R$ that are not detected or less abundant in 184^S cells. IP, immunoprecipitation.

guished by 2DIEF, using an amphotric carrier with a linear pH range of 3 to 10 (Fig. 6). The dominant isoform of p27 in G_0 184^S cells (form 1) was the least negatively charged isoform, migrating close to the predicted IEF point for p27 of 6.5, with others at pI's of 6.24 (form 2), 5.95 (form 3), 5.58 (form 6), and 5.48 (form 7). TGF-β-resistant $184A1L5^R$ cells showed a different pattern of p27 isoforms in G_0. Two major isoforms were seen at pI 6.5 and 5.95, with three minor forms at 6.24, 5.8 (form 4), and 5.65 (form 5). The isoforms at pI 5.54 and 5.48 observed in 184 were not detected in $184A1L5^R$ cells. Forms 1 to 3 were common to both cell lines, as they comigrated when immunoprecipitates from each line were mixed prior to 2DIEF; however, their relative abundance differed significantly between the two cell types. p27 form 1 was the dominant isoform in 184^S cells, while the relative abundance of isoform 3 (pI 5.95) was greater in $184A1L5^R$ cells. With phosphatase treatment prior to 2DIEF, most p27 migrated as form 1, with a small amount migrating as form 2 (Fig. 6) (8). This small amount of form 2 remaining may result from an incomplete phosphatase reaction. However, ^{32}P-orthophosphate labeling showed that forms 1 and 2 did not incorporate any radioactivity (data not shown). While form 1 probably represents unphosphorylated p27, form 2 may represent posttranslational modification (such as acetylation, myristylation, or glycosylation) other than phosphorylation. Taken together, these data suggest that isoforms 3 to 7 seen on 2DIEF are phosphoforms of p27.

Phosphorylation of cyclin E1-bound p27 differs from that of cyclin D1-bound p27. While the levels of cyclin D1 were similar in the sensitive and resistant cells, more p27 was bound to cyclin D1 complexes in proliferating $184A1L5^R$ than in 184^S cells (Fig. 3). Moreover, TGF-β did not inhibit the assembly of newly synthesized p27, cyclin D1, and cdk4 into complexes in $184A1L5^R$ cells (Fig. 2). Since the mobilities of cyclin D1-bound and cyclin E1-bound p27 on SDS-PAGE gels differ (Fig. 3C), suggesting a different p27 phosphorylation state, we performed 2DIEF analysis on cyclin D1-bound p27 from mid-G_1 cells and cyclin E1-bound p27 from G_0-arrested cells using the nonlinear gradient pH 3 to 10 to ascertain whether the "assembly form" of p27 bound to cyclin D1 might differ in its phosphorylation from that bound to cyclin E1. The phosphorylation of cyclin E1-bound p27 (Fig. 7A) differed from that in cyclin D1 complexes (Fig. 7B). Phosphatase treatment confirmed that the different p27 forms were due to phosphorylation (Fig. 7A and data not shown).

Although there was only a minor difference in the phosphorylation patterns of cyclin D1-bound p27 between the two lines, Fig. 3 demonstrated a twofold increase in the total amount of cellular p27 bound to cyclin D1 in $184A1L5^R$ cells compared to the amount in 184^S cells. Taken together, these data suggest that there may be a constitutive alteration of p27 phosphorylation that shifts it toward the cyclin D1-cdk assembly phosphoform in the TGF-β-resistant cells. In addition, there was a subtle difference in phosphorylation patterns of cyclin-bound p27 between $184A1L5^R$ and 184^S cells. Certain cyclin E1-bound phosphoforms detected in $184A1L5^R$ cells were not

seen in cyclin E1 complexes in 184^S cells (arrows, Fig. 7A). The most abundant cyclin D1-bound p27 isoform in 184^S cells was the most hypophosphorylated, and the three most negatively charged (most strongly phosphorylated) p27 isoforms detected in $184A1L5^R$ cells were not detected in cyclin D1 complexes in 184^S cells (arrows, Fig. 7B). These data may reflect constitutive activation of certain p27 phosphorylation events in $184A1L5^R$ cells.

DISCUSSION

Earlier investigation of TGF-β effects in these HMECs suggested a defect in p27 function (35). TGF-β increased p15 protein stability and its association with cdk4 and cdk6 and inhibited assembly of cyclin D1-p27-cdk4 and -cdk6 complexes, while p27 accumulated in cyclin E1-cdk2 in 184^S cells but not in resistant $184A1L5^R$ cells (35). Moreover, cdk4-cyclin D1-KIP complexes from $184A1L5^R$ lysates were resistant to dissociation by p15 in vitro (35). These data led us to postulate that movement of p27 out of cyclin D1-cdk4 complexes might be necessary to allow p15 to displace cyclin D1 and inhibit cdk4 and that altered posttranslational modification of p27 may prevent its dissociation from cdk4 complexes and abrogate sensitivity to G_1 arrest by TGF-β.

Several observations in TGF-β-resistant $184A1L5^R$ cells suggest aberrant p27 regulation. The increased activities of cyclin E1- and cyclin A-dependent kinases and the increased CAK activation of cyclin E1-bound cdk2 are consistent with defective KIP function in $184A1L5^R$ cells. There was more p27 bound to cyclin E1 in proliferating $184A1L5^R$ cells, but p27 binding did not appear to have the same inhibitory consequences as in 184^S cells. Indeed, p27 from proliferating $184A1L5^R$ cells was associated with histone H1 kinase activity, while essentially none was detected in p27 complexes from proliferating 184^S cells. p27 itself is not a kinase. The p27-immunoprecipitable kinase activity detected in $184A1L5^R$ cells may arise through the dissociation of cyclin E1-cdk2 from p27 in vitro after immunoprecipitation. Alternatively, p27 from the resistant cells may bind cyclin E1-cdk2 with an altered conformation that allows both CAK access to cdk2 and cdk2 activity. The observation of p27-associated kinase activity is not without precedent (8, 21, 43). It was recently found that breast cancer cells resistant to antiestrogen-mediated G_1 arrest showed altered p27 phosphorylation, p27-associated kinase activity, and reduced inhibitory function in vitro (8). p27 is a key mediator of G_1 arrest by TGF-β and by antiestrogens, and, thus, it is not surprising that its function is altered in cells resistant to these different forms of G_1 arrest.

In the resistant cells, altered p27 regulation was also manifested by the localization of p27 in both the nucleus and cytoplasm, in contrast to the largely nuclear localization of p27 in 184^S cells. The expression of stable cytoplasmic p27 observed in some primary cancers (40) and in malignantly transformed cell lines (31) could reflect similar perturbations of the cell cycle-regulatory machinery. The strong stable cytoplasmic expression of p27 in $184A1L5^R$ cells suggests either a dissociation of export and degradation (51) or impaired import of newly synthesized p27. It is not clear whether or how the increased cytoplasmic localization of p27 in the $184A1L5^R$ cells is linked to its increased association with cyclin D1 or with its reduced affinity for cyclin E1-cdk2.

In vitro assays indicated a reduced p27 affinity for cyclin E1-cdk2 in G_0-arrested $184A1L5^R$ cells. p27 from $184A1L5^R$ bound and inhibited less recombinant cyclin E1-cdk2 in vitro than did p27 from 184^S. Phosphatase treatment of the p27 prior to cyclin E1-cdk2 inhibition assays abolished the inhibitory activity detected in both cell types (not shown), suggesting that certain p27 phosphorylation events are required for cyclin E1-cdk2 inhibition. Overexpression of c-*myc* has been shown to induce a heat-labile factor that binds p27 and inhibits its association with cyclin E-cdk2 (53). However, the differences in p27 inhibitory activity could not be attributed to sequestration by cyclin D1 or a heat-labile inhibitor of p27 in $184A1L5^R$ cells, since they persisted after boiling and, thus, more likely reflect the differences observed in p27 phosphorylation. While a heat-stable protein could reassociate with p27 and prevent its binding and inhibition of cyclin E1-cdk2 in these assays, the relative rarity of heat-stable cellular proteins mitigates against this.

The reduced ability of p27 from G_0 $184A1L5^R$ to bind and inhibit cyclin E1-cdk2 was associated with an altered p27 phosphorylation profile. 2DIEF identified several p27 isoforms. The dominant form 1 (70% of total) in 184^S cells migrated with the predicted p27 IEF point of 6.5. This hypophosphorylated pI 6.5 isoform represented only 40% of the p27 from $184A1L5^R$ cells. Whether the different p27 isoforms seen on 2DIEF reflect combinations of multiple phosphorylation events or specific changes in single phosphorylation sites is under investigation.

p27 function changes during G_0-to-S-phase progression. p27 is a potent inhibitor of cyclin E1-cdk2 in G_0- and in TGF-β-arrested cells. In quiescence or in TGF-β-arrested HMECs, newly synthesized cyclin D1 fails to bind cdk4. This is not likely attributable to the low abundance of cyclin D1 in quiescence, since cyclin D1 is clearly synthesized in G_0 HMECs and since even ectopically overexpressed cyclin D1 fails to assemble with cdk4 in quiescent fibroblasts (26). The data of Matsushime et al. (26) suggested that a growth factor-stimulated event is required for cyclin D1-cdk4 assembly. Indeed, subsequent work showed that cyclin D1 overexpression mediates p27-cyclin D1-cdk4 complex formation only when coexpressed with an activated MEK, suggesting that MEK-dependent effects may facilitate p27-cyclin D1-cdk4 assembly (6). p27 may require posttranslational modification during G_0-to-G_1 progression in order to function in cyclin D-cdk assembly. We have shown that the pattern of p27 phosphorylation differs when it is bound to inactive cyclin E1-cdk2 in G_0 from that present in cyclin D1-cdk complexes in mid-G_1. Thus, as cells move from G_0 into G_1, p27 acquires the ability to function as a cyclin D1-cdk assembly factor in association with changes in its phosphorylation.

In the $184A1L5^R$ cells, approximately twofold more cellular p27 was detected in cyclin D1-complexes than in 184^S cells. This twofold increase in cyclin D1 binding could potentially be significant, since changes of this magnitude in the amount of p27 accessible for cyclin E-cdk2 binding can have significant effects on cyclin E-cdk2 activity. Reynisdottir et al. showed that a two- to threefold increase in p27 was sufficient to fully saturate cyclin E, leading to G_1 arrest (34). However, there was no reduction in p27 binding to cyclin E1 in asynchronous

184A1L5^R cells; indeed, it was modestly increased. Moreover, immunoprecipitated p27 from resistant cells contained associated histone H1 kinase activity. These observations raise the possibility that the changes in p27 phosphorylation that allow its cyclin D-cdk assembly function in mid-G_1 may occur in association with a reduced ability to inhibit cyclin E complexes. The p27-associated kinase detected in 184A1L5^R cells may reflect kinase-active p27-cyclin-cdk2 complexes or kinase complexes containing p27 that readily dissociate in vitro. There is some experimental evidence to suggest that p27 may exist transiently in a cyclin E-bound, noninhibitory conformation in vivo in G_1 (8, 54). Unfortunately, the low abundance of cyclin E1-bound p27 in mid-G_1 precluded analysis of its phosphorylation status by 2DIEF. Constitutive activation of pathways leading to increased assembly of p27 into cyclin D1-cdk4 complexes may also lead to a change in the inhibitory action of p27 toward cyclin E-cdk2 in the 184A1L5^R cells. Alternatively, the pathways that regulate p27-cyclin D1-cdk4 assembly and p27's cyclin E-cdk2 inhibitory function may be independent of each other, and both may be altered in the TGF-β-resistant cells.

In the G_0 184A1L5^R cells, the reduced ability to bind stably to cyclin E1-cdk2 was associated with altered cellular p27 phosphorylation. T187 phosphorylation of p27 by cyclin E-cdk2 (36, 54) occurs near the G_1-to-S-phase transition (3) and allows p27 recognition by the ubiquitin ligase SCF^{Skp2} complex (Skp2, Cul1, and Skp1) involved in its proteolysis (3, 49, 52). T187 phosphorylation is minimal in G_0, and it does not reduce p27's affinity for cyclin E1-cdk2 in vitro (36, 54) (B. Amati, personal communication). Thus, phosphorylation of sites other than T187 must affect p27 function in 184A1L5^R cells. Although serine 10 appears to be a major p27 phosphorylation site in cells arrested by p27 transfection, mutation of serine 10 did not detectably alter the inhibitory activity of p27 toward cyclin E-cdk2 in vitro (18). Thus, serine 10 phosphorylation may not be relevant to the poor cyclin-E-cdk2 inhibitory function of p27 in 184A1L5^R cells.

Since the assembly and activation of cyclin D-cdk complexes (2, 5, 21) precede activation of cyclin E1-cdk2 during normal G_1 progression, kinases other than cyclin E-cdk2 may phosphorylate p27 and condition it to function as a cyclin D-cdk assembly factor. The noncatalytic function of cyclin D-cdk complexes to titrate p27 may not be exclusively dependent on the abundance of D-type cyclins but is also actively regulated through p27 phosphorylation. As noted earlier, overexpression of activated MEK, together with cyclin D1, can lead to sequestration of p27 into cyclin D1-cdk4 and activate cyclin E-cdk2 though loss of p27 binding (6). Although mitogen-activated protein kinase activation occurs early and is required for G_1-to-S-phase progression in HMECs, the increased assembly of p27 in cyclin D1 complexes in 184A1L5^R cells could not be attributed to increased mitogen-activated protein kinase activation (J. Liang and J. M. Slingerland, unpublished data).

Constitutive *ras* activation has been shown to increase p27 phosphorylation, leading to both a reduced affinity of p27 binding to cdk2 in vitro and to p27 degradation. In human cancer-derived lines, oncogenic activation of receptor tyrosine kinase pathways (23) or of *ras* (1, 19, 50) may lead to TGF-β resistance through accelerated p27 proteolysis or reduced cdk-inhibitory function. Only the latter of these effects is observed in the TGF-β-resistant 184A1L5^R line. In proliferating 184A1L5^R cells, the equilibrium of p27 binding was shifted, with more p27 bound to cyclin D1 than in 184^S cells. Moreover, p27 from the resistant cells showed reduced inhibitory activity against cyclin E1-cdk2. These data suggest a model in which TGF-β modulates the posttranslational regulation of p27, converting it from a factor with high affinity for cyclin D1-cdk4 to a form that binds with high affinity and inhibits cyclin E1-cdk2. In the resistant 184A1L5^R cells, p27 phosphorylation was shifted, allowing increased p27 binding to and assembly of cyclin D1-cdk complexes and the presence of cdk2 bound p27 with reduced affinity for or inhibitory action on cyclin E-cdk2. In the TGF-β-resistant 184A1L5^R cells, constitutive activation of one or more mitogenic pathways may alter p27 phosphorylation, causing failure of p27 to dissociate from cyclin D1-cdk4 and preventing p15 from displacing cyclin D1 and inhibiting cdk4 in response to TGF-β. These changes in p27 function that contribute to TGF-β resistance are likely secondary to alterations in mitogenic signaling pathways in the 184A1L5^R cells. Moreover, the effects of TGF-β on the cell cycle are pleiotropic. While our data suggest a role for deregulated p27 function in the resistant cells, other changes in cell cycle effectors likely contribute to the resistance phenotype. Elucidation of the signal transduction pathways whose activation regulates these events in the 184A1L5^R model may shed light not only on mechanisms of TGF-β resistance but may also reveal the pathways whose activation leads to p27 phosphorylation, acquisition of cyclin D1-cdk assembly function, and the subsequent degradation of p27 during G_1-to-S-phase progression in normal cells.

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REFERENCES

1. **Aktas, H., H. Cai, and G. M. Cooper.** 1997. Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27^{KIP1}. Mol. Cell. Biol. 17:3850–3857.
2. **Cariou, S., J. C. Donovan, W. M. Flanagan, A. Milic, N. Bhattacharya, and J. M. Slingerland.** 2000. Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells. Proc. Natl. Acad. Sci. USA 97:9042–9046.
3. **Carrano, A. C., E. Eytan, A. Hershko, and M. Pagano.** 1999. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. 1:193–199.
4. **Catzavelos, C., N. Bhattacharya, Y. C. Ung, J. A. Wilson, L. Roncari, C. Sandhu, P. Shaw, H. Yeger, I. Morava-Protzner, L. Kapusta, E. Franssen, K. I. Pritchard, and J. M. Slingerland.** 1997. Decreased levels of the cell-cycle inhibitor p27Kip1 protein: prognostic implications in primary breast cancer. Nat. Med. 3:227–230.
5. **Cheng, M., P. Olivier, J. A. Diehl, M. Fero, M. F. Roussel, J. M. Roberts, and C. J. Sherr.** 1999. The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J. 18:1571–1583.
6. **Cheng, M., V. Sexl, C. J. Sherr, and M. F. Roussel.** 1998. Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). Proc. Natl. Acad. Sci. USA 95:1091–1096.
7. **Donovan, J., and J. Slingerland.** 2000. Transforming growth factor-beta and breast cancer: cell cycle arrest by transforming growth factor-beta and its disruption in cancer. Breast Cancer Res. 2:116–124.
8. **Donovan, J. C., A. Milic, and J. M. Slingerland.** 2001. Constitutive MEK/

MAPK activation leads to p27Kip1 deregulation and antiestrogen resistance in human breast cancer cells. J. Biol. Chem. 276:40888–40895.
9. **Draetta, G., and J. Eckstein.** 1997. Cdc25 protein phosphatases in cell proliferation. Biochim. Biophys. Acta 1332:M53–M63.
10. **Dulic, V., E. Lees, and S. I. Reed.** 1992. Association of human cyclin E with a periodic G1-S phase protein kinase. Science 257:1958–1961.
11. **Dumont, N., and C. L. Arteaga.** 2000. Transforming growth factor-beta and breast cancer tumor promoting effects of transforming growth factor-beta. Breast Cancer Res. 2:125–132.
12. **Florenes, V. A., N. Bhattacharya, M. R. Bani, Y. Ben-David, R. S. Kerbel, and J. M. Slingerland.** 1996. TGF-beta mediated G1 arrest in a human melanoma cell line lacking p15INK4B: evidence for cooperation between p21Cip1/WAF1 and p27Kip1. Oncogene 13:2447–2457.
13. **Hannon, G. J., and D. Beach.** 1994. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature 371:257–261.
14. **Hengst, L., V. Dulic, J. M. Slingerland, E. Lees, and S. I. Reed.** 1994. A cell cycle-regulated inhibitor of cyclin-dependent kinases. Proc. Natl. Acad. Sci. USA 91:5291–5295.
15. **Hengst, L., and S. I. Reed.** 1996. Translational control of p27Kip1 accumulation during the cell cycle. Science 271:1861–1864.
16. **Hosobuchi, M., and M. R. Stampfer.** 1989. Effects of transforming growth factor beta on growth of human mammary epithelial cells in culture. In Vitro Cell Dev. Biol. 25:705–713.
17. **Iavarone, A., and J. Massague.** 1997. Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. Nature 387:417–422.
18. **Ishida, N., M. Kitagawa, S. Hatakeyama, and K. Nakayama.** 2000. Phosphorylation at Serine 10, a major phosphorylation site of p27Kip1, increases its protein stability. J. Biol. Chem. 275:25146–25154.
19. **Kawada, M., S. Yamagoe, Y. Murakami, K. Suzuki, S. Mizuno, and Y. Uehara.** 1997. Induction of p27Kip1 degradation and anchorage independence by Ras through the MAP kinase signaling pathway. Oncogene 15:629–637.
20. **Koff, A., M. Ohtsuki, K. Polyak, J. M. Roberts, and J. Massague.** 1993. Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-beta. Science 260:536–539.
21. **LaBaer, J., M. D. Garrett, L. F. Stevenson, J. M. Slingerland, C. Sandhu, H. S. Chou, A. Fattaey, and E. Harlow.** 1997. New functional activities for the p21 family of CDK inhibitors. Genes Dev. 11:847–862.
22. **Laiho, M., J. A. DeCaprio, J. W. Ludlow, D. M. Livingston, and J. Massague.** 1990. Growth inhibition by TGF-beta linked to suppression of retinoblastoma protein phosphorylation. Cell 62:175–185.
23. **Lane, H. A., I. Beuvink, A. B. Motoyama, J. M. Daly, R. M. Neve, and N. E. Hynes.** 2000. ErbB2 potentiates breast tumor proliferation through modulation of $p27^{Kip1}$-Cdk2 complex formation: receptor overexpression does not determine growth dependency. Mol. Cell. Biol. 20:3210–3223.
24. **Lauper, N., A. R. Beck, S. Cariou, L. Richman, K. Hofmann, W. Reith, M. M. Slingerland, and B. Amati.** 1998. Cyclin E2: a novel CDK2 partner in the late G1 and S phases of the mammalian cell cycle. Oncogene 17:2637–2643.
25. **Massague, J., and Y. G. Chen.** 2000. Controlling TGF-beta signaling. Genes Dev. 14:627–644.
26. **Matsushime, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr, and J.-Y. Kato.** 1994. D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell. Biol. 14:2066–2076.
27. **Melchior, F., and L. Gerace.** 1998. Two-way trafficking with Ran. Trends Cell Biol. 8:175–179.
28. **Millard, S. S., J. S. Yan, H. Nguyen, M. Pagano, H. Kiyokawa, and A. Koff.** 1997. Enhanced ribosomal association of p27(Kip1) mRNA is a mechanism contributing to accumulation during growth arrest. J. Biol. Chem. 272:7093–7098.
29. **Montagnoli, A., F. Fiore, E. Eytan, A. C. Carrano, G. F. Draetta, A. Hershko, and M. Pagano.** 1999. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev. 13:1181–1189.
30. **Nakayama, K., N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii, and D. Y. Loh.** 1996. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell 85:707–720.
31. **Orend, G., T. Hunter, and E. Ruoslahti.** 1998. Cytoplasmic displacement of cyclin E-cdk2 inhibitors p21Cip1 and p27Kip1 in anchorage-independent cells. Oncogene 16:2575–2583.
32. **Pagano, M., S. W. Tam, A. M. Theodoras, P. Beer-Romero, G. Del Sal, V. Chau, P. R. Yew, G. F. Draetta, and M. Rolfe.** 1995. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 269:682–685.
33. **Polyak, K., J. Y. Kato, M. J. Solomon, C. J. Sherr, J. Massague, J. M. Roberts, and A. Koff.** 1994. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. 8:9–22.
34. **Reynisdottir, I., K. Polyak, A. Iavarone, and J. Massague.** 1995. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev. 9:1831–1845.
35. **Sandhu, C., J. Garbe, N. Bhattacharya, J. Daksis, C.-H. Pan, P. Yaswen, J. Koh, J. M. Slingerland, and M. R. Stampfer.** 1997. Transforming growth factor β stabilizes $p15^{INK4B}$ protein, increases $p15^{INK4B}$-cdk4 complexes, and inhibits cyclin D1/cdk4 association in human mammary epithelial cells. Mol. Cell. Biol. 17:2458–2467.
36. **Sheaff, R. J., M. Groudine, M. Gordon, J. M. Roberts, and B. E. Clurman.** 1997. Cyclin E-CDK2 is a regulator of p27Kip1. Genes Dev. 11:1464–1478.
37. **Sherr, C. J.** 1994. G1 phase progression: cycling on cue. Cell 79:551–555.
38. **Sherr, C. J., and J. M. Roberts.** 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. 9:1149–1163.
39. **Sherr, C. J., and J. M. Roberts.** 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 13:1501–1512.
40. **Singh, S. P., J. Lipman, H. Goldman, F. H. Ellis, L. Aizenman, M. G. Cangi, S. Signoretti, D. S. Chiaur, M. Pagano, and M. Loda.** 1998. Loss or altered subcellular localization of p27 in Barrett's associated adenocarcinoma. Cancer Res. 58:1730–1735.
41. **Slingerland, J. M., L. Hengst, C.-H. Pan, D. Alexander, M. R. Stampfer, and S. I. Reed.** 1994. A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor β-arrested epithelial cells. Mol. Cell. Biol. 14:3683–3694.
42. **Solomon, M. J., and P. Kaldis.** 1998. Regulation of CDKs by phosphorylation. Results Probl. Cell Differ. 22:79–109.
43. **Soos, T. J., H. Kiyokawa, J. S. Yan, M. S. Rubin, A. Giordano, A. DeBlasio, S. Bottega, B. Wong, J. Mendelsohn, and A. Koff.** 1996. Formation of p27-CDK complexes during the human mitotic cell cycle. Cell Growth Differ. 7:135–146.
44. **Stampfer, M.** 1985. Isolation and growth of human mammary epithelial cells. J. Tissue Cult. Methods 9:107–115.
45. **Stampfer, M. R., and J. C. Bartley.** 1985. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proc. Natl. Acad. Sci. USA 82:2394–2398.
46. **Stampfer, M. R., A. Bodnar, J. Garbe, M. Wong, A. Pan, B. Villeponteau, and P. Yaswen.** 1997. Gradual phenotypic conversion associated with immortalization of cultured human mammary epithelial cells. Mol. Biol. Cell 8:2391–2405.
47. **Stampfer, M. R., C.-H. Pan, J. Hosoda, J. Bartholomew, J. Mendelsohn, and P. Yaswen.** 1993. Blockage of EGF receptor signal transduction causes reversible arrest of normal and immortal human mammary epithelial cells with synchronous re-entry into the cell cycle. Exp. Cell Res. 208:175–188.
48. **Stampfer, M. R., P. Yaswen, M. Alhadeff, and J. Hosoda.** 1993. TGF beta induction of extracellular matrix associated proteins in normal and transformed human mammary epithelial cells in culture is independent of growth effects. J. Cell. Physiol. 155:210–221.
49. **Sutterluty, H., E. Chatelain, A. Marti, C. Wirbelauer, M. Senften, U. Muller, and W. Krek.** 1999. p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. Nat. Cell Biol. 1:207–214.
50. **Takuwa, N., and Y. Takuwa.** 1997. Ras activity late in G_1 phase required for $p27^{kip1}$ downregulation, passage through the restriction point, and entry into S phase in growth factor-stimulated NIH 3T3 fibroblasts. Mol. Cell. Biol. 17:5348–5358.
51. **Tomoda, K., Y. Kubota, and J. Kato.** 1999. Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1. Nature 398:160–165.
52. **Tsvetkov, L. M., K. H. Yeh, S. J. Lee, H. Sun, and H. Zhang.** 1999. p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. Curr. Biol. 9:661–664.
53. **Vlach, J., S. Hennecke, K. Alevizopoulos, D. Conti, and B. Amati.** 1996. Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. EMBO J. 15:6595–6604.
54. **Vlach, J., S. Hennecke, and B. Amati.** 1997. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. EMBO J. 16:5334–5344.
55. **Warner, B. J., S. W. Blain, J. Seoane, and J. Massagué.** 1999. Myc downregulation by transforming growth factor β required for activation of the $p15^{Ink4b}$ G_1 arrest pathway. Mol. Cell. Biol. 19:5913–5922.

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Non-malignant and Tumor-derived Cells Differ in Their Requirement for $p27^{Kip1}$ in Transforming Growth Factor-β-mediated G_1 Arrest*

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Transforming growth factor β (TGF-β) induces G_1 arrest in susceptible cells by multiple mechanisms that inhibit the G_1 cyclin-dependent kinases (Cdks), including Cdk2, Cdk4, and Cdk6. TGF-β treatment of early passage finite lifespan human mammary epithelial cells (HMECs) led to an accumulation of $p27^{Kip1}$ in cyclin E1-Cdk2 complexes and kinase inhibition. The requirement for p27 in the G_1 arrest by TGF-β was assessed by transfection of antisense p27 (ASp27) oligonucleotides into TGF-β-treated HMECs. Despite a reduction in total and cyclin E-Cdk2 bound p27 after ASp27 transfection, HMECs remained arrested in the G_1 phase. Maintenance of the G_1 arrest was accompanied by increased association of the Cdk inhibitor $p21^{WAF-1/Cip-1}$ and the retinoblastoma family member $p130^{Rb2}$ in cyclin E1-Cdk2 complexes along with kinase inhibition. In contrast to the findings in HMECs, p27 was essential for G_1 arrest by TGF-β in two tumor-derived lines. ASp27 transfection into two TGF-β-responsive, cancer-derived lines was not associated with increased compensatory binding of p21 and p130 to cyclin E1-Cdk2, and these cell lines failed to maintain G_1 arrest despite the continued presence of TGF-β. Progressive cell cycle deregulation leading to impaired checkpoint controls during malignant tumor progression may alter the role of p27 from a redundant to an essential inhibitor of G_1-to-S phase progression.

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TGF-β[1] mediates effects on diverse cellular processes such as proliferation, growth, and differentiation via cell surface receptors that in turn regulate the activity of SMAD transcription factors (reviewed in Ref. 1). In many normal cell types, including epithelial and melanocytic cells, TGF-β has a potent antiproliferative effect. In contrast to nontransformed cells, cancer-derived lines show reduced antiproliferative responses to TGF-β or have lost this response altogether (2). In most cases, the loss of TGF-β responsiveness occurs without inactivation of TGF-β receptors or the SMADs. Cell cycle deregulation is believed to contribute to the resistance of malignant cells to G_1 arrest by TGF-β (reviewed in Ref. 3).

TGF-β induces cell cycle arrest in the G_1 phase via a number of pathways that lead ultimately to inhibition of the G_1 cyclin-dependent kinases (Cdks). The Cdks are key mediators of progression through the cell cycle and are regulated by phosphorylation, cyclin binding, and by the binding of Cdk inhibitory proteins (reviewed in Ref. 4). During G_1-to-S phase progression, the D-type cyclins bind Cdk4 and Cdk6 and the E-type cyclins bind Cdk2, contributing to kinase activation and G_1-to-S phase progression. The G_1 phosphatase, Cdc25A, plays an essential role in Cdk activation by the removal of inhibitory phosphates from Cdk2 (5) and possibly also from Cdks 4 and Cdk6 (6). Cdc25A may be transcriptionally up-regulated by c-Myc (7). Two families of Cdk inhibitory proteins oppose Cdk activation. $p21^{WAF1/Cip-1}$, $p27^{Kip1}$, and $p57^{Kip2}$ belong to the kinase inhibitory protein (KIP) family and contribute to the inhibition of cyclin E1-Cdk2 complexes in the G_1 phase. $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$ belong to the inhibitors of Cdk4 family and act to inhibit Cdk4 and Cdk6 (reviewed in Ref. 4).

AQ: B

In several cell types, including human mammary epithelial cells (HMECs) and mink lung epithelial cells, TGF-β induces and stabilizes the p15 protein, which leads to its binding to and inhibition of Cdk4 and Cdk6 complexes (8–10). TGF-β also causes the accumulation of p27 in cyclin E1-Cdk2 complexes leading to Cdk2 inhibition (11, 12). Changes in several essential cell cycle regulators cooperate to induce TGF-β arrest, including down-regulation of c-Myc (11, 13), Cdc25A (6), and cyclin D1 and, in some cell types, up-regulation of p21 (3). Deregulation of various cell cycle targets including cyclin and Cdk overexpression, Cdk inhibitor inactivation, and Myc or Cdc25A overexpression are believed to contribute to TGF-β resistance in cancer (3).

Although mouse embryonic fibroblasts (MEFs) from $p27^{-/-}$ mice retain TGF-β sensitivity (14), several studies have indicated an association between altered p27 regulation and the development of TGF-β resistance. Our previous work showed that the acquisition of TGF-β resistance in human mammary epithelial cells was associated with altered phosphorylation, altered Cdk inhibitory activity, and cytoplasmic mislocalization of the p27 protein (15). Although *p27* gene mutations are rare in human tumors, increased proteasomal degradation of p27 is observed in a number of cancers, including breast, colon, and prostate, and the reduced levels are associated with poor patient prognosis (reviewed in Refs. 16 and 17). Relatively little is known about the compensatory mechanisms invoked by a

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[1] The abbreviations used are: TGF-β, transforming growth factor-β; Cdk, cyclin-dependent kinase; KIP, kinase inhibitory protein; HMEC, human mammary epithelial cells; MEFs, mouse embryonic fibroblasts; ASp27, antisense p27.

nontransformed cell after a reduction in p27 protein levels, although a few reports support a role for compensation by other Cdk inhibitors to maintain normal cell cycle control. For example, in serum-starved $p27^{-/-}$ mouse embryonic fibroblasts (MEFs), the accumulation of the retinoblastoma family member p130^{Rb2} in cyclin E-Cdk2 complexes compensated for p27 loss and enabled cells to undergo proliferative arrest in the G_1 phase (18).

The present study investigated the requirement for p27^{Kip1} in maintaining G_1 arrest by TGF-β in finite lifespan HMECs and in cancer-derived lines. Using antisense p27 oligonucleotides to inhibit p27 expression, we show that HMECs, but not the tumor cell lines, maintain G_1 arrest after p27 down-regulation via a compensatory accumulation of p21 and p130 in cyclin E-Cdk2 complexes. These data suggest that p27 is required to maintain TGF-β arrest in these malignant lines but has a redundant function in the finite lifespan HMEC that can be compensated for by other Cdk inhibitory mechanisms.

EXPERIMENTAL PROCEDURES

Cell Culture—The derivation and culture of normal finite lifespan human mammary epithelial cells from reduction mammoplasty has been described previously (10, 19). The WM35 human melanoma line was derived from a radial growth phase melanoma and was kindly provided by Dr. M. Herlyn (Wistar Institute, Philadelphia, PA). Cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (Hyclone Laboratories) (20). MCF-10A cells are spontaneously immortalized human mammary epithelial cells derived from a patient with benign breast disease as described previously (21). MCF-10A cells were kindly provided by Dr. F.-F. Liu (Ontario Cancer Institute) and cultured in MCDB 170 medium with additives as described previously (6). MCF-7 cells (22) were grown in improved-modified essential medium-option Zn^{2+} supplemented with insulin and 5% fetal calf serum. HMECs and WM35 cells were treated with 10 ng/ml TGF-β; MCF-10A and MCF-7 cells were treated with 100 ng/ml TGF-β purchased from R & D Systems (Minneapolis, MN).

Flow Cytometric Analysis—Cells were pulse-labeled with 10 μM bromodeoxyuridine for 2 h and then fixed, stained with anti-bromodeoxyuridine-conjugated fluorescein isothiocyanate (BD Biosciences) and counterstained with propidium iodide as described previously (23). Cell cycle analysis was carried out on a BD Biosciences FACScan and Cell Quest Software.

Immunoblotting—Cell lysis and immunoblotting were performed as described previously (10). Equal protein loading was verified by blotting for β-actin. To assay cyclin E1 complexes, cyclin E1 was immunoprecipitated from 600 μg of protein lysate with monoclonal anti-cyclin E mAb172. To assay Cdk6 immunoprecipitates, Cdk6 was immunoprecipitated from 300 μg of protein lysate. Immunoprecipitates were resolved, transferred, and blotted with the appropriate antibody for detection of associated proteins. Antibody alone controls were run along side immunoprecipitates. The data presented are representative of at least three repeat assays.

Antibodies—Monoclonal antibodies to p27 and p130 were from BD Transduction Laboratories. Antibodies to p21, Cdk2, Cdk6, Cdc25A, and c-Myc were obtained from Santa Cruz Biotechnology; to cyclin D1 (DCS-6) from Neomarkers; to PSTAIRE from S. Reed (The Scripps Research Institute, La Jolla, CA), to cyclin E1 (mAbs E12 and E172) and p15 (JC-6) from E. Harlow (Massachusetts General Hospital, Boston, MA); and to β-actin from Sigma.

Antisense Oligonucleotide Transfection—184HMEC, MCF-10A, WM35, or MCF-7 cells were treated with TGF-β for 24 h followed by antisense or missense p27 oligonucleotide transfection using 2.5 μg/ml cytofectin G3815 (Gilead Scientific, Foster City, CA) for 6 h as described previously (24) in the presence of TGF-β followed by replacement with fresh media containing TGF-β. Flow cytometry and protein analysis was performed immediately after transfection and at 24 h thereafter. For 184 and MCF-10A cells, neither varying the oligonucleotide concentration from 5 nM to 1, 10, 25, 50, or 120 nM nor varying the transfection time from 6 h to 1, 3, 4, 10, or 24 h abrogated the G_1 arrest by TGF-β.

Cyclin-dependent Kinase Assays—Cyclin E1 was immunoprecipitated and reacted with [γ-^{32}P]ATP and histone H1 as described previously (12, 25). Radioactivity incorporated in the histone H1 substrate was quantitated using an Amersham Biosciences PhosphorImager and ImageQuant software. Radioactivity incorporated in control nonspecific mouse polyclonal IgG immunoprecipitates was subtracted from test kinase values.

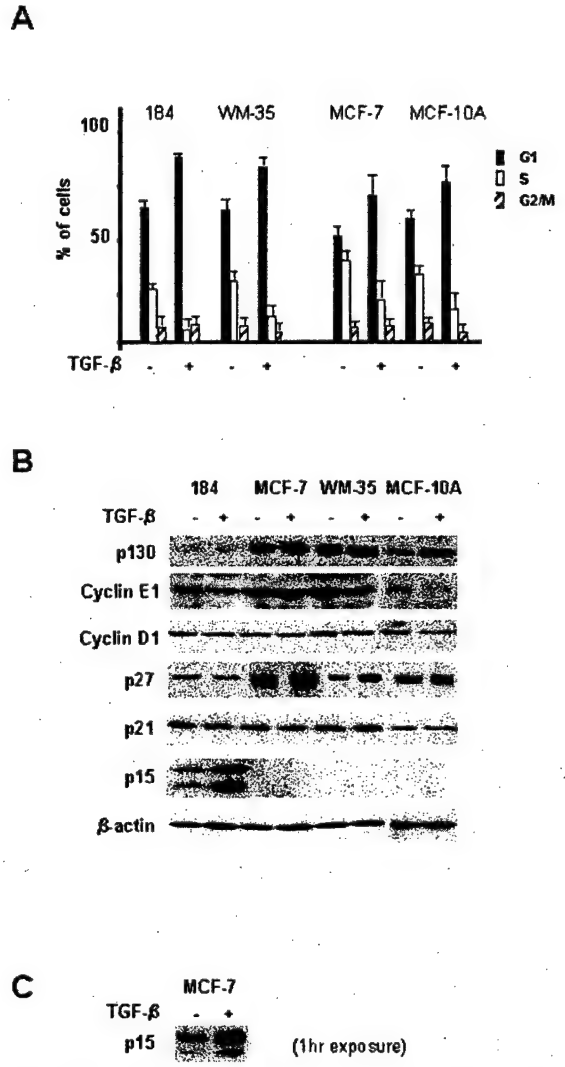

FIG. 1. **Effects of TGF-β on the cell cycle profile and G_1 regulatory proteins.** *A*, flow cytometric analysis of asynchronously proliferating (−) and 48-h TGF-β-treated (+) 184, WM35, MCF-10A, and MCF-7 cells. *B*, cell lysates from the treatment groups in *A* were analyzed by Western blotting using the indicated antibodies. *C*, 1-h exposure of the film shown in *B* of p15 protein in MCF-7 in the absence (−) and after 48 h of TGF-β treatment (+). 184 and WM35 were treated with 10 ng/ml TGF-β; MCF-10A and MCF-7 were treated with 100 ng/ml TGF-β.

RESULTS

TGF-β Effects on Cell Cycle Profiles—We compared the TGF-β responsiveness of human mammary epithelial cells (184 HMEC, passage 11), WM35, MCF-10A, and MCF-7 cells (Fig. 1A). 184 HMEC are a finite lifespan mammary epithelial strain, MCF-10A is a spontaneously immortalized non-malignant breast epithelial cell line, MCF-7 is a malignant breast cancer line, and WM35 is a malignant melanoma cell line. Cells were treated for 48 h in the absence (−) or presence (+) of TGF-β (Fig. 1A). 184 and WM35 cells had similar sensitivity to TGF-β, undergoing G_1 arrest with an ~80% reduction in the proportion of cells in S phase after 48 h of TGF-β treatment (10 ng/ml). The MCF-10A and MCF-7 cell lines were less sensitive than the 184 HMEC or WM35, but both underwent partial G_1 arrest with 100 ng/ml TGF-β with more than 50% reduction in the proportion of cells in S phase.

TGF-β Effects on Cyclin and Cdk Inhibitor Levels—The levels of the relevant G_1 cyclins, Cdks, and Cdk inhibitors were assayed by Western analysis in 184, MCF-7, WM35, and MCF-10A cells in the absence (−) or after a 48-h exposure (+) to TGF-β (Fig. 1*B*). Cyclin D1 levels were similar in asynchronously proliferating 184, MCF-7, and WM35 cells and slightly lower in MCF-10A cells. Cyclin E1 levels were ~2.5-fold greater in the untreated cancer-derived MCF-7 and WM35 cells compared with the 184 and MCF-10A HMEC. Cyclin E1 and cyclin D1 levels showed no consistent alteration by TGF-β in repeat assays in 184, MCF-7, and WM35 cells. In MCF-10A, however, TGF-β decreased cyclin D1 and cyclin E1 levels by up to 4-fold. The levels of p27 were higher in the cancer-derived lines, with asynchronously proliferating MCF-7 and WM35 cells having p27 levels approximately 15- and 3-fold greater, respectively, than asynchronously proliferating 184 HMEC and MCF-10A cells. TGF-β treatment did not alter p27 protein levels in the HMEC, but p27 levels rose by ~1.5-fold in the MCF-7 line, 3-fold in WM35, and 5-fold in MCF-10A. Total p21 levels were similar in 184, MCF-7, and WM35, with reduced levels in MCF-10A. p21 levels were unchanged in the 184 and MCF-7 cells after 48 h of TGF-β treatment. The WM35 cells showed a transient increase in p21 levels at 18–30 h of TGF-β treatment followed by a return to similar levels as in the asynchronous population by 48 h. MCF-10A cells showed a modest decrease in p21 levels at the 48-h time point. p15 levels were much higher in the 184 HMEC compared with the MCF-7 line; WM35 and MCF-10A are p15-null (6, 20). TGF-β treatment of 184 HMEC led to a 3-fold increase in p15 levels. In MCF-7 cells, p15 could not be detected in the short exposure times (3–5 min) that were used to detect p15 from the 184 HMEC. However, a longer exposure of the film (1 h) showed that p15 levels increased by 1.5–2-fold in TGF-β-treated MCF-7 cells (Fig. 1*C*).

Given that the $p130^{Rb2}$ protein, like $p21^{WAF-1/Cip-1}$ and $p27^{Kip1}$, has a Cdk inhibitory domain and can also accumulate in and inhibit Cdk complexes (18), p130 protein levels were assayed in asynchronously proliferating and TGF-β-treated cells by Western analysis. p130 levels were much lower in HMEC compared with the cancer-derived lines (Fig. 1*B*). TGF-β modestly increased p130 levels (less than 1.5-fold) in 184 HMEC and also increased p130 in MCF-10A. p130 levels were not affected by TGF-β in the tumor-derived lines. Equal loading was verified by β-actin.

TGF-β Effects on Cyclin-Cdk Composition and Activities—The levels of p21, p27, p130, and Cdk2 in cyclin E1 complexes were assayed after immunoprecipitation of equivalent levels of cyclin E1 from asynchronously proliferating and TGF-β-treated 184, MCF-7, WM35, and MCF-10A cells (Fig. 2*A*). Cyclin E1-bound Cdk2 levels were similar and were not altered by TGF-β in repeat assays in all four cell types. Cyclin E1-bound p21 levels were unaltered in 184 HMEC after TGF-β treatment, but TGF-β treatment of MCF-7, WM35, and MCF-10A cells led to a modest increase (1.5–2-fold) in p21 binding to cyclin E1. p27 increased in cyclin E1 complexes in all four cell types after TGF-β treatment (Fig. 2*B*). Paradoxically, asynchronously proliferating cancer-derived lines showed a greater amount of cyclin E1-bound p27 than did HMECs. Cyclin E1-bound p27 levels were ~8–15 times higher in proliferating MCF-7 and WM35 than in 184 HMEC. Cyclin E1-bound p21 was also 2-fold higher in MCF-7, WM35, and MCF-10A lines than in 184 HMEC. p130 was detected in cyclin E1 complexes in both asynchronously proliferating and TGF-β-treated cells (Fig. 2*A*). Although the total p130 levels were much lower in 184 HMEC and MCF-10A cells compared with the cancer-derived lines (see again Fig. 1*B*), the levels of cyclin E1-bound p130 were ~5–10-fold higher in 184 HMEC than in the cancer derived lines. p130 binding to cyclin E1-Cdk2 was only modestly increased by TGF-β in 184, MCF-7, and WM35. TGF-β treatment of MCF-10A led to a 3-fold increase in cyclin E1-bound p130.

The histone H1 kinase activity of cyclin E1-complexes shown in Fig. 2*A* was assayed as described under "Experimental Procedures" (Fig. 2*B*). Although equal amounts of cyclin E1 were precipitated, cyclin E1-associated kinase activities in MCF-7, WM35, and MCF-10A were 4–5 times greater than that in the asynchronously proliferating 184 HMEC. On repeat assays, TGF-β treatment of the 184 HMEC and MCF-10A led to a nearly complete (>95%) inhibition of cyclin E1-associated kinase activity. WM35 cells also showed 90% reduction in kinase activity associated with the G_1 arrest. Cyclin E1-associated kinase activity in the MCF-7 cells was reduced by TGF-β by ~60%. Representative histone H1 kinase autoradiography is shown in the *inset*.

Loss of p15 Up-regulation by TGF-β in MCF-7 and WM35—We assayed levels of p15 and cyclin D1 present in Cdk6 complexes from asynchronously proliferating and TGF-β-treated cells (Fig. 2*C*). Despite similar total cyclin D1 and Cdk6 in 184, MCF-7, and WM35 cells, more cyclin D1 was bound to Cdk6 in asynchronously proliferating MCF-7 and WM35 than in 184 HMEC (see Figs. 1*B* and 2*C*). Cdk6-bound cyclin D1 levels in MCF-10A were intermediate between that observed in 184 and MCF-7 or WM35. TGF-β caused a 2–3-fold reduction in the levels of Cdk6-bound cyclin D1 in 184 cells, whereas Cdk6-bound cyclin D1 association was not notably reduced by TGF-β in the MCF-7, WM35, and MCF-10A cells. Cdk6-bound p15 was significantly higher in asynchronously proliferating 184 than MCF-7 cells. The level of p15 bound to Cdk6 increased ~5-fold after TGF-β arrest of the 184 HMEC. In contrast, p15 levels were significantly reduced and Cdk6-bound p15 levels did not increase after TGF-β treatment of MCF-7, even on longer exposure of the Cdk6-associated p15 blot (Fig. 2*D*). WM35 and MCF-10A cells lack p15 because of a biallelic loss of the *p15* gene (6, 20).

Increased c-Myc and Cdc25A Levels in Cancer-derived Lines—The WM35 and MCF-7 cancer-derived lines in our study showed a number of differences in the regulation of p27, cyclin D1, cyclin E1, and p15 compared with the 184 HMEC. The cyclin E1-associated kinase activities were increased despite the presence of increased cyclin E1-Cdk2 bound p27 in these complexes in both asynchronously proliferating and TGF-β-treated MCF-7 and WM35 lines. We observed increased cyclin D1 bound to Cdk6 complexes and a failure to accumulate p15 in Cdk6 complexes after TGF-β treatment of the cancer-derived lines. These observations prompted us to assay the levels of c-Myc, because c-Myc has been shown to interfere with p27 function at many levels and to repress *p15* induction (26–29). c-Myc may also transactivate the *Cdc25A* gene (7), whose product is an important activator of Cdk2 (7) and whose down-regulation plays an important role in G_1 arrest by TGF-β (6).

The levels of c-Myc and Cdc25A proteins were assayed in the cancer-derived MCF-7 and WM35 cell lines and in the 184 HMEC (Fig. 2*E*). c-Myc levels were 5–10-fold greater in the asynchronously proliferating cancer-derived MCF-7 and WM35 lines compared with 184 HMEC; Cdc25A levels were approximately 15–20-fold higher. c-Myc and Cdc25A levels in MCF-10A were intermediate between those in 184 and in the two cancer-derived lines. After TGF-β treatment, Cdc25A levels were reduced ~5-fold in 184, MCF-7, and MCF-10A, and by ~2-fold in WM35. Although Cdc25A levels were reduced by TGF-β in all four cell types, the residual amount of Cdc25A protein present in TGF-β-treated cells differed importantly. c-Myc and Cdc25A levels remaining in the TGF-β-treated can-

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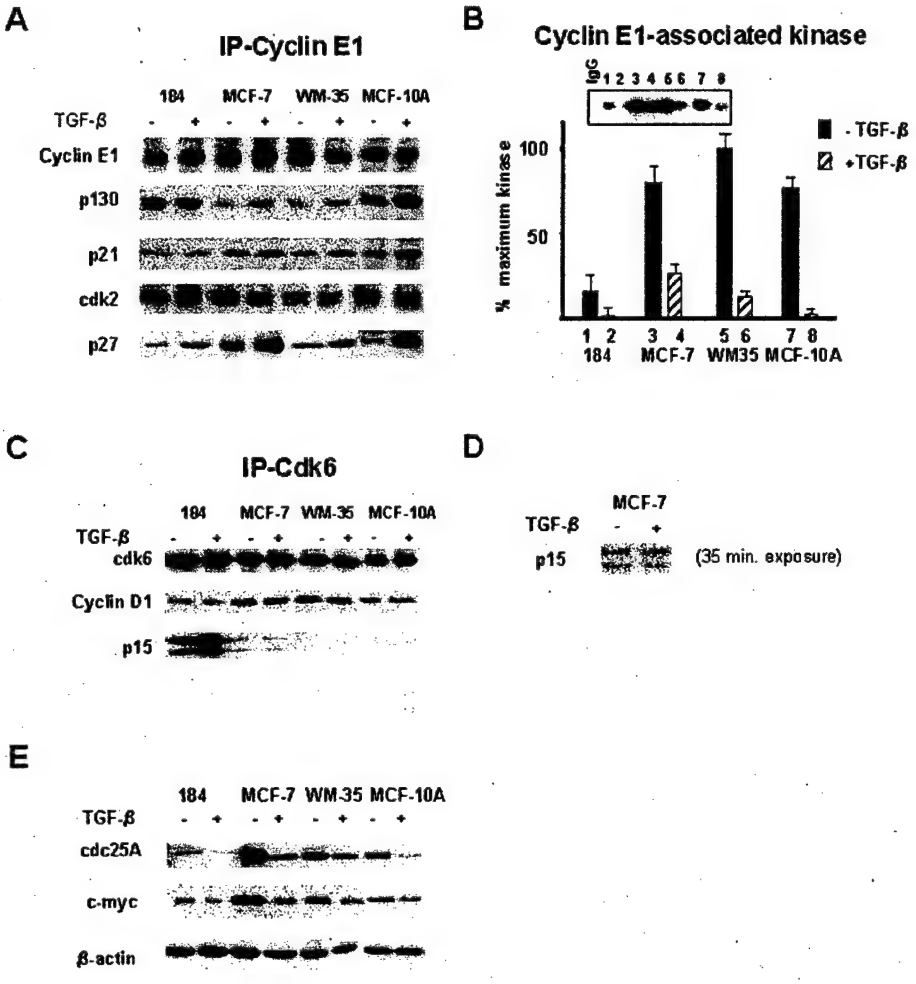

FIG. 2. **G_1 Cdk complexes are regulated differently in normal and cancer-derived lines.** *A*, cyclin E1 immunoprecipitates (*IP*) from asynchronously proliferating and TGF-β-treated cells were resolved and assayed for associated p21, p27, p130, and Cdk2. *B*, cyclin E1 immunoprecipitates were also analyzed for associated histone H1 kinase activity. The *inset* shows radioactivity in nonspecific IgG control and in *reactions 1–8*. The activities are graphed as a percentage maximum for *lanes 1–8* after subtraction of the background activity in the IgG control. *C*, Cdk6 immunoprecipitates from asynchronously proliferating TGF-β-treated cells were resolved and assayed for associated cyclin D1 and p15. *D*, Cdk-6-associated p15 after 35-min film exposure. *E*, Western analysis of c-Myc and Cdc25A levels in asynchronously proliferating and TGF-β-treated cells.

cer lines were 5–10-fold higher than in the TGF-β-treated HMEC.

ASp27 Activates Cyclin E1-Cdk2 and Abrogates TGF-β-induced G_1 Arrest in Tumor-derived Lines but Not in Nonmalignant Lines—p27 was discovered as a mediator of cyclin E-Cdk2 inhibition and G_1 arrest by TGF-β (11, 12). However, in different cell types, other changes in G_1 regulators seem to contribute to TGF-β mediated arrest (reviewed in Ref. 3). To specifically address the requirement for p27 in TGF-β-mediated G_1 arrest, we tested whether the antisense-mediated inhibition of p27 expression would abrogate TGF-β arrest in 184 HMEC, WM35, MCF-7, and MCF-10A cells. Cells were treated with TGF-β for 36 h followed by a 6-h transfection with antisense-p27 (ASp27), mismatch oligonucleotides, or lipid alone as controls. Fresh media containing TGF-β was added back after transfection. Cell cycle and protein analysis were performed immediately after the transfection and at 24 h. p27 protein levels were reduced by 3–5-fold after ASp27 transfection and levels remained low 24 h after transfection (Fig. 3*A*). p27 levels in the control and mismatch oligonucleotide-transfected groups were similar. The transfection did not alter protein levels of other G_1 regulators examined including p21, p130, cyclin E1, cyclin D1, Cdk2, and Cdk6 (data not shown).

ASp27 caused TGF-β-arrested MCF-7 and WM35 to re-enter the cell cycle but had no such effect on arrested 184 HMEC or MCF-10A cells. Flow cytometric analysis at 24 h showed that ASp27 transfection led to a decrease in the proportion of cells in G_1 and an increase in the proportion in S phase in the tumor-derived WM35 and MCF-7 lines (Fig. 3*B*). Approximately 25–30% of these cells were in S phase 24 h after transfection compared with only 9–15% for the lipid and mismatch controls. Equal amounts of cyclin E1 were immunoprecipitated from ASp27-treated and from mismatch p27 or lipid controls, and histone H1 kinase activities were assayed. Reactivation of cyclin E1-dependent kinase accompanied cell cycle re-entry after antisense-mediated inhibition of p27 expression in TGF-β-treated MCF-7 and WM35 cells (shown for WM35 in Fig. 3*C*).

In contrast, the cell cycle profiles of the finite lifespan 184 HMEC and the immortalized MCF-10A line were not altered by ASp27 transfection. Repeat assays showed that the cyclin E1-associated kinase remained inhibited in G_1 arrested ASp27-transfected 184 HMEC and MCF-10A, as it did in TGF-β-treated lipid and mismatch controls (Fig. 3*C*, data shown for 184 HMEC). Thus, p27 is required to maintain G_1 arrest by TGF-β in these tumor-derived lines but not in finite lifespan or immortal, nonmalignant mammary epithelial cells.

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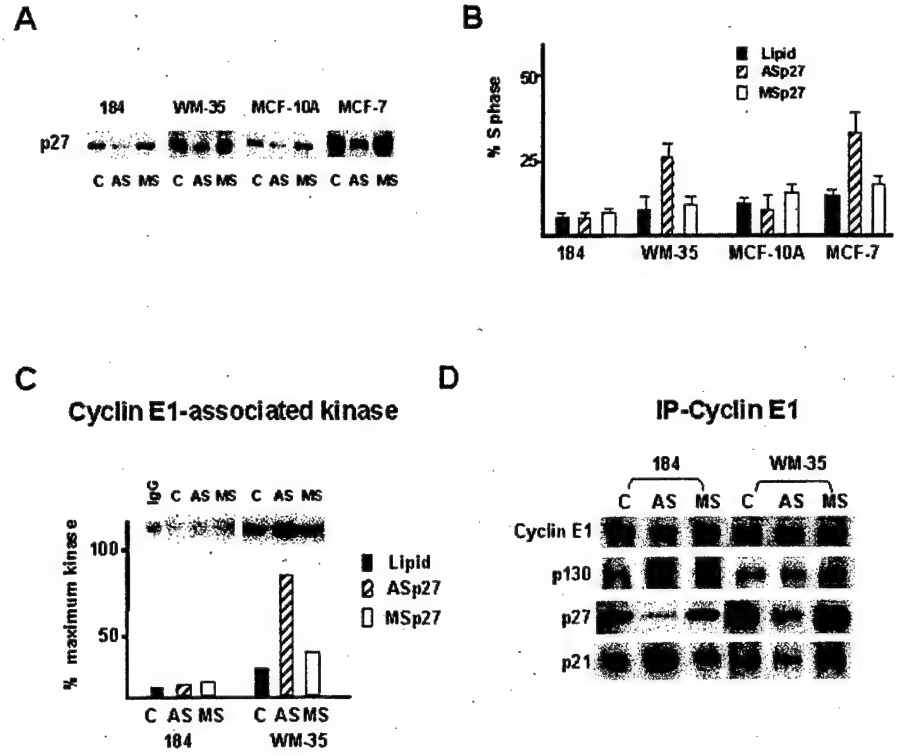

FIG. 3. **Maintenance of TGF-β-mediated G_1 arrest in the HMEC, but not malignant tumor-derived lines after p27 down-regulation.** The indicated cells were treated with TGF-β for 36 h, followed by a 6-h transfection with lipid only (*C*), antisense p27 oligonucleotides (*AS*), and mismatch (*MS*) oligonucleotides. The levels of p27 (*A*) and cell cycle profile (*B*) were assayed 24 h after transfection. The cyclin E1-associated kinase activities (*C*) and cyclin E1-bound p21, p27, p130, and Cdk2 (*D*) were assayed in 184 and WM35 cells 24 h after transfection.

The failure of antisense p27-transfected 184 HMEC and MCF-10A cells to re-enter the cell cycle was not caused by toxicity, because replacement of the TGF-β-containing media with complete media (no TGF-β) led to cell cycle re-entry (data not shown). Because early passage and late passage HMEC differ in their responsiveness to TGF-β (30), we repeated the ASp27 transfection experiments with mid-passage (passage 15) and late passage (passage 20) 184 HMEC. Regardless of passage, 184 HMEC maintained G_1 arrest in the presence of TGF-β. The failure of the malignant tumor-derived lines to maintain G_1 arrest after ASp27 was unlikely to have been caused by differences in the intrinsic TGF-β sensitivity of the cell types. The finite lifespan 184 HMEC and malignant WM35 lines had similar TGF-β sensitivity, as did the nonmalignant MCF-10A and malignant MCF-7 cells lines, yet only 184 and MCF-10A cells maintained arrest by TGF-β after p27 down-regulation.

Increased Association of p21$^{WAF-1/Cip1}$ and p130^{Rb2} Contribute to Cyclin E1-Cdk2 Inhibition in ASp27-treated HMEC but Not in Cancer-derived Lines—To investigate mechanisms contributing to maintenance of TGF-β arrest in 184 HMEC and immortal MCF-10A, despite the antisense-mediated decrease in p27 expression, we assayed the levels of p21, p27, and p130 bound to cyclin E1 in ASp27-transfected, TGF-β-treated cells. In all four cell types, ASp27 treatment significantly reduced the levels of p27 in cyclin E1 complexes (shown for 184 HMEC and WM35 in Fig. 3*D*). In the 184 HMEC, the reduction in cyclin E1-bound p27 was associated with a ~3–5-fold increase in cyclin E1-bound p21 and a 5–10-fold increase in the level of p130 in the cyclin E1 complexes (Fig. 3*D*). TGF-β-treated MCF-10A also showed increased cyclin E1-bound p130 and p21 after antisense-mediated loss of p27 and maintained the G_1 arrest (not shown). In the cancer-derived MCF-7 and WM35 lines, there was no increased p130 association with cyclin E1 complexes; cyclin E1-bound p21 decreased in ASp27-treated cells compared with the lipid and mismatch cells and ASp27 led to cell cycle re-entry (shown for WM35, Fig. 3*D*).

DISCUSSION

Loss of sensitivity to the growth inhibitory effect of TGF-β is common in human tumor-derived cell lines and is thought to contribute to malignant tumor progression (31). Although increased p27 proteolysis and TGF-β resistance have both been shown to occur early in tumorigenesis, previous work has not provided a causal link between p27 deregulation and loss of G_1 arrest by TGF-β during oncogenic progression. With the exception of the increased size of *p27*-null mice compared with wild-type mice, the relative absence of alterations in development, differentiation, and cell cycle control in *p27*-null mice suggests that compensation by other cell cycle regulators may occur in the absence of p27 (11, 14, 32, 33). Indeed, mouse embryonic fibroblasts (MEFs) obtained from *p27*-null mice retain sensitivity to many growth inhibitory stimuli, including TGF-β (14). The present study demonstrates that p27 is an essential mediator of G_1 arrest by TGF-β in two malignant lines, the MCF-7 breast cancer cell line and the WM35 melanoma line. Antisense-mediated inhibition of p27 expression led to cyclin E1-Cdk2 reactivation and cell cycle re-entry. However, p27 was not essential for G_1 arrest by TGF-β in two non-tumor-derived cell types, the finite lifespan 184 HMEC and the immortalized MCF-10A line. In these cells, a compensatory increase in binding of p21$^{WAF-1/CIP-1}$ and p130^{Rb2} to cyclin E1-Cdk2 complexes seems to contribute to maintenance of the G_1 arrest.

A number of studies using oncogene-transformed or cancer-derived cell lines support the notion that p27 loss or deregulation is associated with impaired TGF-β arrest response. Overexpression of the Bcr-Abl kinase in human M07 cells and murine Ba/F3 cells led to the proteosomal degradation of p27,

which was associated with TGF-β resistance (34). Oncogenic *ras* activation led to cytoplasmic mislocalization of p27 and to TGF-β resistance in epithelial cell lines (35). In the WM35 and 184HMEC used in this study, we reported recently that overexpression of activated protein kinase B impairs TGF-β responsiveness, at least in part through protein kinase B-mediated phosphorylation of p27, leading to its cytoplasmic mislocalization (36). Furthermore, *E1A* overexpression in mink lung epithelial cells caused TGF-β resistance, and these cells failed to accumulate p27 in cyclin E1-Cdk2 complexes in response to TGF-β (37). In the present study, we observed differences in p27 regulation in the cancer-derived lines compared with two human mammary epithelial cell types, 184 HMEC and MCF-10A. Asynchronously proliferating cancer-derived lines, especially MCF-7, had a paradoxically high amount of p27 present in cyclin E1-cdk2 complexes. In addition, cyclin E1-Cdk2 complexes from both cancer-derived lines had higher kinase activities than asynchronous 184 HMEC, despite more cyclin E1-bound p27 and p21. These data suggest that the Cdk inhibitory activity of the KIPs may be impaired in MCF-7 and WM35 cells. In the context of functional KIP deregulation, even a modest loss of p27 via antisense might have a critical effect because compensatory action by p21 may be impaired.

The p130^{Rb2} protein may play an important compensatory role in maintenance of checkpoints after p27 loss in several cell types, including epithelial cells, as we report here, and fibroblasts. In *p27$^{-/-}$* MEFs, the accumulation of p130 in cyclin E-Cdk2 complexes compensated for the absence of p27 and contributed to Cdk2 inhibition and G_1 arrest after either pharmacologic phosphatidylinositol 3-kinase inhibition or serum starvation (18, 38). Other studies support a role for p130 in the proliferative arrest by TGF-β. Herzinger *et al.* (39) showed an accumulation of p130 in E2F complexes and repression of E2F regulated genes during TGF-β arrest of human keratinocytes. We detected p130 in cyclin E1-Cdk2 complexes from both asynchronously proliferating and TGF-β-treated cells. In all cells assayed, TGF-β treatment induced a modest yet similar increase in the levels of cyclin E1-bound p130. Surprisingly, despite 15–20-fold higher p130 protein levels in the two malignant tumor-derived lines than in the nonmalignant cells, the levels of p130 bound to cyclin E1-cdk2 were 5–10-fold less in these cancer-derived lines than in the HMEC. Thus, mechanisms that regulate p130 binding to cyclin E1-Cdk2 complexes may differ between the HMEC and cancer-derived lines.

p130 deregulation has been observed, and may have independent prognostic value, in several types of human cancers (40–42). Altered p130 regulation has been reported in the context of altered p27 regulation and may contribute to loss of responses to antiproliferative stimuli. For example, the viral E1A protein can bind and inactivate both p27 and p130, and *E1A* overexpression leads to TGF-β resistance (37). In addition, *p27$^{-/-}$* lymphocytes, which express lower p130 levels than *p27$^{-/-}$* MEFs, fail to commit to G_1 arrest after serum starvation (18). Thus, deregulation of both p130 and p27 potentially lead to a loss of normal proliferative control during tumor progression. Future studies may elucidate whether p130 deregulation further stratifies for poor patient outcome among patients whose tumors show reduced p27.

Our data support the notion that deregulation of multiple G_1 cell cycle regulators may be required before cells lose responsiveness to antiproliferative effects of TGF-β. Deregulation of several G_1 regulators may ultimately be required before p27 becomes essential for G_1 arrest by TGF-β. p15 has been shown to cooperate with p27 in G_1 arrest by TGF-β (8). p15 is not required for G_1 arrest by TGF-β because both MCF-10A (6) and WM35 (20) retain TGF-β responsiveness despite a lack of p15 expression. Moreover, p15 loss *per se* does not make cells dependent on p27 for TGF-β-mediated G_1 arrest. Although both MCF-10A and WM35 are p15-deficient, ASp27 abrogated TGF-β arrest in only the WM35 cells and not the MCF-10A cells. Thus, cell cycle inhibitory pathways activated by TGF-β in p15-deficient MCF-10A that compensate for ASp27-mediated loss do not seem to be functional in WM35. One of these may be the compensatory increase in p130 binding to Cdk2. The disruption of both p130 and p15 regulation in cancers may alter the role of p27 from a redundant to essential mediator of G_1 arrest by TGF-β.

c-Myc plays an important role in the regulation of many G_1 cell cycle proteins, including cyclin E1, p27, p21, p15, and Cdc25A. Moreover, c-Myc overexpression causes TGF-β resistance (26). In MCF-7, the elevated c-Myc levels may contribute to the loss of induction of p15 by TGF-β. Increased c-Myc may also be linked to the impaired anti-proliferative role of p130 (43, 44) and could contribute to the increased expression of Cdc25A (7) in MCF-7 and WM35.

The increased Cdc25A levels in WM35 and MCF-7 may contribute to the increased cyclin E1-Cdk2 activities observed in these lines. Cdc25A down-regulation contributes to G_1 arrest by TGF-β (6). Although TGF-β reduced Cdc25A levels in all of the cell types, MCF-7 and WM35 had significantly higher residual Cdc25A levels remaining after 48 h of TGF-β treatment than did 184 HMEC and MCF-10A. The higher residual levels of the Cdk2 activator Cdc25A in the TGF-β arrested cancer cells may make them more susceptible to cyclin E1-Cdk2 activation after antisense-mediated p27 loss. Cangi *et al.* (45) have shown increased mortality in breast cancer patients whose tumors expressed both elevated Cdc25A and low p27. In addition, there was a positive correlation between Cdk2 activity and Cdc25A expression in the breast cancers studied. Increased Cdc25A expression and activity would oppose the cyclin E1-Cdk2 inhibitory function of p27. In cancers with Cdc25A overexpression, maintenance of p27 expression and function may become critical for continued responsiveness to such antiproliferative stimuli as TGF-β.

In summary, our data support the notion that a reduction in p27 levels may contribute significantly to the loss of normal responsiveness to growth inhibitory stimuli during cancer progression. Importantly, the reduction in p27 levels alone may be insufficient to disrupt cell cycle arrest responses when other cdk inhibitory mechanisms are functional. Our antisense experiments suggest that normal mammary epithelial cells maintain their antiproliferative responses at least in part through activation of the cdk inhibitory function of p21 and p130 when p27 levels are reduced. Loss of these and other normal checkpoint controls during malignant progression may make p27 essential for G_1 arrest by TGF-β.

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REFERENCES

1. Massague, J. (1998) *Annu. Rev. Biochem.* **67,** 753–791
2. Reiss, M., and Barcellos-Hoff, M. H. (1997) *Breast Cancer Res. Treat.* **45,** 81–95
3. Donovan, J., and Slingerland, J. (2000) *Breast Cancer Res.* **2,** 116–124
4. Sherr, C. J., and Roberts, J. M. (1999) *Genes Dev.* **13,** 1501–1512
5. Hoffmann, I., Draetta, G., and Karsenti, E. (1994) *EMBO (Eur. Mol. Biol. Organ.) J.* **13,** 4302–4310
6. Iavarone, A., and Massague, J. (1997) *Nature* **387,** 417–422
7. Galaktionov, K., Chen, X., and Beach, D. (1996) *Nature* **382,** 511–517
8. Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995) *Genes Dev.* **9,** 1831–1845
9. Hannon, G. J., and Beach, D. (1994) *Nature* **371,** 257–261
10. Sandhu, C., Garbe, J., Daksis, J., Pan, C. H., Bhattacharya, N., Yaswen, P., Koh, J., Slingerland, J., and Stampfer, M. R. (1997) *Mol. Cell. Biol.* **17,** 2458–2467
11. Koff, A., Ohtsuki, M., Polyak, K., Roberts, J. M., and Massague, J. (1993) *Science* **260,** 536–539

12. Slingerland, J. M., Hengst, L., Pan, C. H., Alexander, D., Stampfer, M. R., and Reed, S. I. (1994) *Mol. Cell. Biol.* **14,** 3683–3694
13. Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, M. R., Munger, K., Howley, P. M., and Moses, H. L. (1990) *Cell* **61,** 777–785
14. Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., and Loh, D. Y. (1996) *Cell* **85,** 707–720
15. Ciarallo, S., Subramaniam, V., Hung, W., Lee, J. H., Kotchetkov, R., Sandhu, C., Milic, A., and Slingerland, J. M. (2002) *Mol. Cell. Biol.* **22,** 2993–3002
16. Slingerland, J., and Pagano, M. (2000) *J. Cell. Physiol.* **183,** 10–17
17. Lee, M. H., and Yang, H. Y. (2001) *Cell. Mol. Life Sci.* **58,** 1907–1922
18. Coats, S., White, P., Fero, M. L., Lacy, S., Chung, G., Randel, E., Firpo, E., and Roberts, J. M. (1999) *Curr. Biol.* **9,** 163–173
19. Stampfer, M. (1985) *J. Tissue Cult. Methods* **9,** 107–115
20. Florenes, V. A., Bhattacharya, N., Bani, M. R., Ben-David, Y., Kerbel, R. S., and Slingerland, J. M. (1996) *Oncogene* **13,** 2447–2457
21. Tait, L., Soule, H. D., and Russo, J. (1990) *Cancer Res.* **50,** 6087–6094
22. Soule, H. D., Vazguez, J., Long, A., Albert, S., and Brennan, M. (1973) *J. Natl. Cancer Inst.* **51,** 1409–1416
23. Petrocelli, T., Poon, R., Drucker, D., Slingerland, J., and Rosen, C. (1996) *Oncogene* **12,** 1387–1396
24. Cariou, S., Donovan, J. C., Flanagan, W. M., Milic, A., Bhattacharya, N., and Slingerland, J. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 9042–9046
25. Dulic, V., Lees, E., and Reed, S. I. (1992) *Science* **257,** 1958–1961
26. Amati, B., Alevizopoulos, K., and Vlach, J. (1998) *Front. Biosci.* **3,** D250–D268
27. Feng, X. H., Liang, Y. Y., Liang, M., Zhai, W., and Lin, X. (2002) *Mol. Cell.* **9,** 133–143
28. Staller, P., Peukert, K., Kiermaier, A., Seoane, J., Lukas, J., Karsunky, H., Moroy, T., Bartek, J., Massague, J., Hanel, F., and Eilers, M. (2001) *Nat. Cell Biol.* **3,** 392–399
29. Warner, B. J., Blain, S. W., Seoane, J., and Massague, J. (1999) *Mol. Cell. Biol.* **19,** 5913–5922
30. Hosobuchi, M., and Stampfer, M. R. (1989) *In Vitro Cell Dev. Biol.* **25,** 705–713
31. Fynan, T. M., and Reiss, M. (1993) *Crit. Rev. Oncog.* **4,** 493–540
32. Fero, M. L., Rivkin, M., Tasch, M., Porter, P., Carow, C. E., Polyak, K., Firpo, E., Tsai, L., Broudy, V., Perlmutter, R. M., Kaushansky, K., and Roberts, J. M. (1996) *Cell* **85,** 733–744
33. Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O., Soares, V. C., Hoffman, E. S., Ono, M., Khanam, D., Hayday, A. C., Frohman, L. A., and Koff, A. (1996) *Cell* **85,** 721–732
34. Jonuleit, T., van der Kuip, H., Miething, C., Michels, H., Hallek, M., Duyster, J., and Aulitzky, W. E. (2000) *Blood* **96,** 1933–1939
35. Liu, X., Sun, Y., Ehrlich, M., Lu, T., Kloog, Y., Weinberg, R. A., Lodish, H. F., and Henis, Y. I. (2000) *Oncogene* **19,** 5926–5935
36. Liang, J., Zubovits, J., Petrocelli, T., Kotchetkov, R., Connor, M., Han, K., Lee, J. H., Ciarallo, S., Beniston R, Franssen, E., and Slingerland, J. (2002) *Nat. Med.*, in press.
37. Mal, A., Poon, R., Howe, P., Toyoshima, H., Hunter, T., and Harter, M. (1996) *Nature* **380,** 262–265
38. Collado, M., Medema, R. H., Garcia-Cao, I., Dubuisson, M. L., Barradas, M., Glassford, J., Rivas, C., Burgering, B. M., Serrano, M., and Lam, E. W. (2000) *J. Biol. Chem.* **275,** 21960–21968
39. Herzinger, T., Wolf, D. A., Eick, D., and Kind, P. (1995) *Oncogene* **10,** 2079–2084
40. Susini, T., Baldi, F., Howard, C. M., Baldi, A., Taddei, G., Massi, D., Rapi, S., Savino, L., Massi, G., and Giordano, A. (1998) *J Clin Oncol* **16,** 1085–1093
41. Tanaka, N., Ogi, K., Odajima, T., Dehari, H., Yamada, S., Sonoda, T., and Kohama, G. (2001) *Cancer* **92,** 2117–2125
42. Massaro-Giordano, M., Baldi, G., De Luca, A., Baldi, A., and Giordano, A. (1999) *Clin. Cancer Res.* **5,** 1455–1458
43. Mateyak, M. K., Obaya, A. J., and Sedivy, J. M. (1999) *Mol. Cell. Biol.* **19,** 4672–4683
44. Queva, C., McArthur, G. A., Ramos, L. S., and Eisenman, R. N. (1999) *Cell Growth Differ.* **10,** 785–796
45. Cangi, M. G., Cukor, B., Soung, P., Signoretti, S., Moreira, G., Jr., Ranashinge, M., Cady, B., Pagano, M., and Loda, M. (2000) *J. Clin. Invest.* **106,** 753–761

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The MAPK pathway: new molecular targets for the therapy of hormone resistant breast cancer

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About seventy percent of newly diagnosed breast cancers express the estrogen receptor (ER) and of these, two thirds will respond to antiestrogen therapies. Millions of breast cancer patients are treated with Tamoxifen worldwide. In spite of the initial efficacy of hormonal therapies, in time, breast cancers progress to a hormone resistant state. A better understanding of how antiestrogens arrest breast cancer growth is a first step in the development of drugs that can overcome hormone resistance.

Tamoxifen causes breast cancer cells to arrest in the G1 phase of the cell cycle. Our data show that the cell cycle inhibitors, p27 and p21, are essential for the clinical efficacy of antiestrogens. p21 and p27 levels increase following treatment with antiestrogen drugs, Tamoxifen or ICI182780, leading to G1 arrest. When p21 or p27 expression was inhibited by antisense, cells whose proliferation had been blocked by Tamoxifen or ICI182780 re-entered the cell cycle. These data demonstrate that depletion of either p21 or p27 can mimic estrogen stimulated cell proliferation and indicate that both of these cell cycle inhibitor proteins are essential mediators of the therapeutic effects of Tamoxifen on breast cancer.

We have observed constitutive activation of the MAPK pathway in hormone resistant, ER positive breast cancer lines. In these lines, inhibition of the MAPK kinase (MEK) restored sensitivity to growth arrest by Faslodex (ICI 182,780) and by Tamoxifen. Antisense inhibition of p27 expression abolished this sensitivity, confirming the importance of p27 in this antiestrogen mediated arrest. Moreover, transfection of constitutively activated MEK into hormone sensitive cells induced loss of p27 and partial resistance to antiestrogen mediated cell cycle arrest. Effects of the MAPK pathway on p27 and p21 function and the therapeutic implications of these observations will be addressed.

Akt/PKB-dependent phosphorylation of p27 activates the cyclin D1/Cdk4 assembly function of p27 and G1 cell cycle progression

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G1 progression in mammalian cells is driven by D and E type cyclin/cdks, whose activation is tightly regulated in response to extra-cellular cues. Cyclin D1 and the cdk inhibitor, $p27^{Kip1}$, are thought to be crucial sensors for positive and negative proliferation signals, respectively. $p27^{Kip1}$ plays a role in maintaining quiescence induced by TGF-β, contact inhibition, or growth factor depletion. In contrast to their growth inhibitory function, Kip family proteins also mediate the assembly of D type cyclin-cdks, that is critical for the activation of the holoenzymes. However, it remains unclear how Kip assembly function is regulated by mitogenic signaling.

We previously demonstrated that the cyclin D1-cdk assembly function of $p27^{Kip1}$ is increased and phosphorylation of $p27^{Kip1}$ is altered in a TGF-β resistant cell line. To explore the potential role of mitogenic PI3K signaling in G1 progression, we examined PI3K/PKB activation across the cell cycle. Activation of Akt/PKB occurs early in G1 and precedes the assembly of cyclin D1/cdk4. Inhibition of PI3K blocks G1/S progression. We present several lines of evidence suggesting that the requirement of PI3K/Akt activation for G1/S progression is at least in part due to its ability to phosphorylate $p27^{Kip1}$ and facilitate $p27^{Kip1}$'s cyclin D1/Cdk4 assembly function. (1) Cyclin D1/cdk4/Kip complex formation is increased in cells expressing constitutively active Akt/PKB (PKB^{DD}). (2) Akt/PKB phosphorylates $p27^{Kip1}$ on T157, and T157A mutant $p27^{Kip1}$ binds to cyclin D1 or cdk4 less efficiently than wild-type p27or T157D mutant $p27^{Kip1}$ *in vivo* and *in vitro*. (3) The $p27^{Kip1}$ released from cellular cyclin D1-cdk complexes can readily mediated assembly of cyclin D1/Cdk4, while p27 released from cdk2 complexes is less effective in cyclin D1-cdk4 assembly assays *in vitro*. (4) *In vitro* phosphorylation of recombinant $p27^{Kip1}$ by Akt/PKB greatly increased its ability to assemble cyclin D1/Cdk4. Moreover, p27 released by boiling from cdk2 complexes acquired competence as a cyclin D1-cdk4 assembly factor following phosphorylation by PKB (5) As revealed by tryptic mapping, a highly phosphorylated site of cyclin D1-bound cellular $p27^{Kip1}$ is only minimally phosphorylated in cdk2-bound $p27^{Kip1}$. This site is also absent in $p27^{Kip1}$ T157A. Thus, Akt/PKB dependent phosphorylation of $p27^{Kip1}$ appears to play a crucial role in early G1 phase to activate the cyclin D1/Cdk4 assembly function of $p27^{Kip1}$ and G1/S progression.